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Effects of diets containing digestion-resistant starch on Wnt pathway control of proliferation and differentiation of the colorectal mucosa

Nicole Cray

Iowa State University

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Effects of diets containing digestion-resistant starch on Wnt pathway control of proliferation and differentiation of the colorectal mucosa

By

Nicole Lynn Cray

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
Elizabeth Whitley, Co-Major Professor
Diane Birt, Co-Major Professor
Kevin Schalinske

Iowa State University

Ames, Iowa

2013

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
</tbody>
</table>

## CHAPTER 1: GENERAL INTRODUCTION
- Introduction          | 1   |
- Thesis Organization    | 3   |
- Literature Review      | 5   |
- References             | 27  |

## CHAPTER 2: Effects of Dietary Resistant Starch on the Wnt Signaling Pathway in Azoxymethane-Damaged Rat Colon
- Abstract               | 35  |
- Introduction           | 36  |
- Materials and Methods  | 40  |
- Results                | 46  |
- Discussion             | 50  |
- Abbreviations          | 54  |
- Acknowledgements       | 55  |
- References             | 55  |
- Figure Captions        | 59  |
- Figures                | 60  |
- Table Captions         | 68  |
- Tables                 | 69  |

## CHAPTER 3: Stearic Acid-Complexed High-Amylose Cornstarch Modulates the Wnt Signaling Pathway in Azoxymethane-Initiated Deep Crypt Epithelial Cells of the Mouse Colon
- Abstract               | 75  |
- Introduction           | 76  |
- Materials and Methods  | 77  |
- Results                | 80  |
- Discussion             | 84  |
- Abbreviations          | 85  |
- Acknowledgements       | 90  |
- References             | 91  |
- Figure Captions        | 91  |
- Figures                | 95  |
- Table Captions         | 96  |
- Tables                 | 100 |

## CHAPTER 4: GENERAL CONCLUSIONS
- 106
LIST OF FIGURES
Figure 1. Chromosomal Instability Pathway (Walther et al 2009) 8
Figure 2. Wnt signaling pathway 11
Figure 3. Starch viewed under polarized light 21
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ABSTRACT

Resistant starch (RS) is not digested in the small intestine and is available for fermentation by the colonic microbiota, producing metabolites including short chain fatty acids (SCFAs). We hypothesized that diets containing resistant starch, serving as a prebiotic, will have inhibitory effects, mediated through the products of microbial fermentation, on the canonical Wnt signaling pathway. To test this hypothesis, we examined the molecular and morphologic effects of diets containing resistant starches on the early stage of colorectal carcinogenesis in azoxymethane-induced rat and mouse models. The first study examined the effects of diets containing naturally occurring resistant starch from Latin American corn strains, GUAT (18.6%) and AR (5.4%), and their hybrid, ARxGUAT (8.6%), for effects on the pre-neoplastic stages of azoxymethane-induced colonic epithelial damage in Fisher 344 rats. Genes of the Wnt signaling pathway were selected and analyzed for expression levels by semi-quantitative, real-time polymerase chain reaction analysis (semi-qRT-PCR). In the second study, the effects of diets including a stearic-acid complexed high-amylose cornstarch (SA-HA7) (31.1%), a high amylose starch (HA7) (19.0%), a chemically modified starch (OS-HA7) (4.7%), were compared with normal corn starch (CS) (3.7%), for the effects on azoxymethane-induced colonic epithelial damage in A/J mice, as tested by mRNA levels of the same genes as the first study. Being able to demonstrate inhibitory effects of resistant starch, on the Wnt pathway will provide a potential means to prevent the initiation and proliferation of colorectal carcinogenesis. In the first study, the GUAT diet did not inhibit development of the putative preneoplastic lesion, aberrant crypt focus (ACF). However, mRNA encoding β-catenin was significantly decreased in AOM-injected rats fed the GUAT diet compared with the saline-injected rats fed the GUAT diet, while no similar decrease was seen in diets low in resistant starch. In the second study, mice fed a diet
containing a high level of resistant starch, SA-HA7, had lower mRNA expression of several targets, with the exception of \(\beta\)-catenin mRNA, relative to the mice fed the control diet. Overall, this suggests a potential ability of resistant starch to regulate \(\beta\)-catenin expression, and thus the Wnt signaling pathway.
CHAPTER 1: GENERAL INTRODUCTION

Introduction

Resistant starch is a starch that escapes digestion in the small intestine and is available for fermentation in the colon (Englyst, 1986). The products of this fermentation by colonic bacteria include short chain fatty acids (SCFAs), molecules that have been shown to have a protective effect on the epithelial cells of the colon (Cummings, et al., 1996). Butyrate, acetate, and propionate are the three primary SCFAs, with butyrate believed to have the greatest effects on the colon, and have been shown to play an important role in inhibiting proliferation and inducing apoptosis in colonic epithelial cells (O'Keefe, et al., 2009).

Resistant starches have been shown, through both human and animal studies, to have many health benefits as a prebiotic, including protection against colonic DNA damage, obesity, diabetes, and chronic inflammatory bowel disease (Jacobasch, et al., 1999; Hasjim, et al., 2010; Lattimer and Haub, 2010). Such benefits in animal studies suggest that there is strong potential for wide-spread health benefits for humans.

Naturally occurring resistant starch has been found in many plants. Carbohydrates are stored in plants in the form of starch, with high concentrations in grains and seeds to provide a source of stored nutritional energy. Chemically, starch molecules are polysaccharides composed of linear or helical amylose molecules or branched amylopectin which are stored in varying concentrations, depending on the relative activities of starch synthase and branching enzymes in each strain of plant. High concentrations of amylose correspond to increased digestion resistance because enzymatic action on a linear molecule occurs more slowly overall than on a branched molecule (Tharanathan, 2002). Five types of RS have currently been categorized, including physically inaccessible starch (RS1), naturally highly resistant starch to $\alpha$-amylase digestion
(RS2), retrograded resistant starch (RS3), chemically modified starch (RS4), and amylase-lipid complexed starch (RS5). Physicochemical modification and conjugation of various molecules are effective ways to increase the resistance of starch granules against enzymatic hydrolysis. A novel starch (SA-HA7) was created by complexing a high-amyllose starch with steric acid (Hasjim, et al., 2010).

The two studies in this thesis examined the inhibitory effect of cooked diets containing the resistant starches, GUAT, SA-HA7, and OS-HA7, on the azoxymethane-damaged colonic epithelium in rodent models. The resistant starches are not always chemically modified; naturally occurring resistant starches are sometimes present in high concentrations in maize, such as the Guatemalan line, GUAT. The starch in kernels of other lines, such as an Argentinean line, AR, has a low level of resistance to digestion and is high-yielding. The variation in resistant starch content comes, in part, from the genetic make-up of the plant and the amylase to amylopectin ratio. For example, corn starch with the sbe1a mutation was reported to alter molecular structures resulting in a better resistance to pancreatic $\alpha$-amylase, $\beta$-amylase, and isoamylase than wild type starch (Xia, et al., 2011). Parental lines, GUAT and AR, were crossed by members of the Resistant Starch PSI group, Dr. Susan Duvick and Dr. Linda Pollak, working in the Department of Agriculture at Iowa State University, in hopes that the best qualities of each parent, high resistance and high yielding, respectively, would be passed down to hybrid, ARxGUAT.

Effects of dietary resistant starch were determined by examining the mRNA expression levels of key genes of the Wnt signaling pathway, as it is an important regulator of proliferation and differentiation in the colonic epithelium. The Wnt pathway regulates proliferation through the influence of Wnt family growth factors and controls migration of epithelial cells up the
colonic crypt. Proliferation ceases after the cells have reached approximately two-thirds of the way up the crypt walls as the cells migrate away from the Wnt signal. This epithelial proliferation and migration controlled by Wnt factors is called the non-canonical Wnt signaling pathway (Schneikert and Behrens, 2007). When this system becomes deregulated (typically through up-regulation), a state designated the canonical Wnt pathway; control of epithelial cell growth is lost, with possible development of colorectal cancer. Resistant starch, and thus the microbial metabolic product of resistant starch fermentation, butyrate, has been hypothesized to be able to induce a hyper-induction of Wnt, initiating apoptosis (Bordonaro, et al., 2008). The initiation of the apoptotic cycle then provides a secondary, “rescue” mechanism for regulating and controlling unregulated tumorigenic cell growth. This hyper-induction has also been shown, through the Notch signaling pathway, to cause post translational modifications of β-catenin. This is hypothesized to inhibit cell proliferation by halting the stem cell and progenitor cell population in the G₀ phase of the cell growth cycle (Kwon, et al., 2011).

The purpose of the studies included in this thesis is to assess the inhibitory effect of various resistant starches on the genetic mechanisms underlying CRC. The hypothesis underpinning these studies is that resistant starch and the microbial metabolic products generated from resistant starch will regulate the Wnt signaling pathway, controlling proliferation of normal and pre-neoplastic (initiated) colonic epithelial cells.

**Thesis Organization**

This thesis examines the inhibitory effect of cooked diets containing high concentrations of resistant starches (ARxGUAT, GUAT, HA7, OS-HA7, and SA-HA7) or highly digestible starches (AR and a commercially available cornstarch) on the canonical Wnt signaling pathway.
of azoxymethane-induced rat and mouse model using $\beta$-catenin as a primary target gene along with other genes and proteins that are involved in colorectal carcinogenesis and serve as indicators of CRC.

I will first review the pertinent literature to examine background concepts and the formulation for hypotheses on which these studies are based. I will then report the results of an investigation evaluating the effects of diets containing starch derived from one of three corn lines (AR, ARxGUAT, and GUAT) and their effects on a pre-neoplastic lesions, ACF (work conducted prior to my arrival at Iowa State University), and mRNA expression of genes of the Wnt signaling pathway with my genetic studies contributing to preexisting lesion studies. In this study, Dr. Linda Pollak and Dr. Duvick in the Department of Agriculture in Iowa State University carried out all corn line crossings. Dr. Yinsheng Zhao assisted with the starch processing, set up the animal model, and analyzed the efficacy of resistant starch on a putative preneoplastic lesion. I analyzed the efficiency of resistant starches on the mRNA expression of key genes in the Wnt signaling pathway of both full-thickness colonic tissue and cells of the colonic epithelial surface and crypt. I also wrote the manuscript for this collective work. In a second manuscript, I will report the effects of diets containing one of three forms of resistant starch (HA7, OS-HA7, and SA-HA7) or control starch on the expression of Wnt signaling genes. Dr. Jay-Lin Jane, Michael Reed, and Yongfeng Ai in the Department of Food Science and Human Nutrition in Iowa State University modified the high-amylose 7 resistant starch to both OS-HA7 and SA-HA7 and analyzed the resistant starch content of the diets in both studies. Bridget Nelson, also of the Department of Food Science and Human Nutrition, and I collectively set up the animal model for this study under the supervision of Dr. Diane Birt and Dr. Elizabeth Whitley of the Department of Food Science and Human Nutrition and Department of Veterinary
Pathology, respectively. Reed Faldet, Kylie Thompson, Dana Pralle, and Rachel Scieszinski assisted with animal care. I analyzed the effectiveness of resistant starches on the mRNA expression of key genes in the Wnt signaling pathway from colonic crypt epithelium. I also wrote the manuscript for this collective work. At the end of the dissertation, a general conclusion will be presented.

**Literature Review**

**Colorectal Cancer (CRC)**

Colorectal cancer is a leading cause of cancer deaths in the world and is the third most prevalent cancer in the United States, affecting men and women equally. The American Cancer Society estimates 102,480 new cases of colon cancer and 40,340 new cases of rectal cancer in 2013. They also estimate that, this year alone, CRC will cause 50,830 deaths (American Cancer Society, 2013). Statistics from a 2008 study by the World Health Organization show that the highest rates of incidence of colorectal cancer are observed in Australia, New Zealand, and Western Europe with the lowest rates observed in Africa and South-Central Asia (GLOBOCAN). The highest rates of mortality are in Central and Eastern Europe with the lowest mortality rate being in Middle Africa (GLOBOCAN). Center, et al. showed that CRC incidence rates are on the rise in countries that are transitioning to becoming fully economically developed countries, affecting both males and females with equal incidence (Center, et al., 2009). This research, based on data from 1983-87 and 1998-2002, also reported a stabilization of CRC incidence among fully developed countries.

Colorectal cancer develops in a series of molecular steps reflected by alterations in morphology along a continuum extending from normal to preneoplastic to neoplastic (benign,
then malignant) and, sometimes, metastatic lesions. Cancer of the colon develops as a consequence of uncontrolled cell growth by the cells of the epithelial lining of the colon or rectum. This neoplasm develops in the epithelial lining of the colon or rectum, with most cancers starting as benign polyps, sometimes eventually progressing to malignant adenocarcinomas, and may grow through some or all of these other layers of tissue that form the wall of the colon. Once this neoplasm invades through the epithelial lining and migrates to the draining lymph node or another organ, it is classified as a metastasis (Faltermann, et al., 1974).

Colorectal cancers can arise through either a sporadic genetic mutation or a hereditary genetic condition. About 94% of CRCs arise through a sporadic mutation, while approximately 6% of CRCs are inherited (Coleman and Tsongalis, 2009). Familial adenomatous polyposis (FAP), the predominant form of the inherited CRCs, is characterized by polyp formation in the colon and upper gastrointestinal tract. Individuals with this condition may also experience desmoid tumors (Kohler, et al., 2009). In both sporadic CRC and FAP, the underlying cause is a mutation in the Adenomatous Polyposis Coli (APC) gene, which is located on human chromosome 5q21 (Groden, et al., 1991; Shibata, 1997; Kohler, et al., 2009). Up to 80% of all sporadic colorectal carcinomas have been shown to have a loss-of-function mutation in the APC gene (Schneikert and Behrens, 2007). APC is known as a tumor-suppressor gene, so a mutation that results in inactivity of the gene product would allow progression toward malignancy (Segditas and Tomlinson, 2006; Schneikert and Behrens, 2007). The disease may then progress through the stages of dysplasia, adenoma, adenocarcinoma, and, finally, in fulminant disease, distant metastasis (Nambiar, et al., 2003).

While familial adenomatous polyposis (FAP) is rare, among the documented cases, there are many genetic variations due to the many possible mutations of APC (Dienz and Clevers,
The most common APC mutation is a deletion of the nucleotide sequence, AAAAG, beginning at codon 1309, a defect which is present in 10% of all FAP cases (Giles, et al., 2003). Many of these mutations affect only one allele, resulting in loss of control of cell proliferation that allows the development of one or more colonic polyps, non-malignant neoplasms also called adenomas. It is not until the second allele becomes mutated that the loss of FAP function allows the development of a cancerous lesion. Adenomas can lead to cancer if left untreated, due to the accumulation over time of this critical second mutation (Giles, et al., 2003; Reya and Clevers, 2005; Segditsas and Tomlinson, 2006; Schneikert and Behrens, 2007; Kohler, et al., 2009). The progression toward cancer starts with crypts of the colon undergoing hyperplasia. A lesion seen in the epithelium in association with CRC, aberrant crypt foci (ACF), is believed to occur commonly in colon cancer patients and may serve as a precursor lesion for CRC (Papanikolaou, et al., 1998). Preventive measures for CRC currently include surveillance measures, such as colonoscopy, to identify and remove pre-malignant lesions. (Schneikert and Behrens, 2007).

**Mechanism of Colon Cancer**

Both sporadic and hereditary forms of colon cancer are believed to follow the same progression from ACF to metastatic carcinoma (Coleman and Tsongalis, 2009). There are three primary molecular pathways through which this progression occurs. They are the Chromosomal Instability Pathway (CIN), the Microsatellite Instability Pathway (MSI), and the CpG Island Methylator Pathway (CIMP). The CIN pathway is the most common pathway for colon cancer progression. This pathway is characterized by gains and losses of large chromosomal regions and neoplastic cells commonly have allelic loss (Figure 1).
Inactivation of tumor-suppressor genes like $APC$ or $p53$ begins this stepwise progression toward colorectal carcinogenesis. Under this circumstance, normal epithelium will undergo de-regulated proliferation and aberrant crypt foci (ACF) may develop. The inactivation of $APC$ results in inactivation in the $Wnt$ signaling pathway. This inactivation of the $Wnt$ pathway leads to translocation of $\beta$-catenin into the nucleus where it progressively accumulates and initiates transcription activation of key genes needed for cell proliferation, differentiation, and migration (Van de Wetering, et al., 2002). $\beta$-Catenin thus plays an important role in enhancing neoplastic progression toward early adenoma. De-regulation of the $Wnt$ signaling pathway is not, however, the only mechanism that affects the progression toward carcinogenesis. Mutations in the $K-ras$ gene, an oncogene frequently activated in cancer, occur during ACF formation shortly following the $APC$ inactivation and also support progression not only to early adenomas, but also to intermediate adenomas (Coleman and Tsongalis, 2009). Inactivation of $SMAD2$ and $SMAD4$, two tumor-suppressor genes, then cause these intermediate-stage adenomas to progress to late-stage adenomas (Sancho, 2007). Adenomas typically develop additional genetic mutations, with
those in \textit{p53} and \textit{DCC} (deleted in colon cancer) being most frequent (Fearon and Jones, 1992). Defects in these additional tumor-suppressor genes provide a further mechanism for progression to malignancy. Mutations in \textit{E-cadherin} will further result in a metastatic carcinoma (Coleman and Tsongalis, 2009).

The Microsatellite Instability pathway (MSI) is characterized by defects in the DNA mismatch repair proteins. A loss of function in any one of these proteins (MLH1, MSH2, MSH6, and PMS2) results in a large number of genetic mutations within any one of the genes discussed above and several others affecting the progression of cancer (Coleman and Tsongalis, 2009).

The CpG Island Methylator Phenotype pathway (CIMP) is characterized by abundant CpG methylation of DNA molecules of neoplastic cells. This hypermethylation leaves genes unable to be transcribed or proteins unable to bind to DNA. The methylation can silence genes which regulate cell proliferation, differentiation, and migration. The methylation can also inactivate genes which are classified as tumor suppressors, leaving them unable to function properly. CIMP does appear to be able to interact with the \textit{Wnt} signaling pathway and to methylate needed regulators of the \textit{Wnt} pathway (Coleman and Tsongalis, 2009).

Together, these three main molecular mechanisms, CIN, MSI, and CIMP, demonstrate the stepwise progression of colorectal carcinogenesis and the important role genetic mutations play in each model. While the chromosomal instability pathway may be the most common, all three mechanisms are important in understanding colorectal cancer. This pathway also underlies the \textit{Wnt} signaling pathway, which is the focus of the research presented in this thesis.

\textit{Wnt} Signaling Pathway
The *Wnt* pathway is involved in many different processes of mammalian development including cell proliferation, differentiation, and epithelial-mesenchymal interaction. These processes contribute to the development and maintenance of many tissues and organs (Moon, et al., 1997; Smalley and Dale, 1999). The *Wnt* pathway controls many of these activities by regulating and acting on the stem cell populations in each of these tissues (Reya and Clevers, 2005; Nusse, 2008). Because the *Wnt* signal acts on the stem cell populations, de-regulation of this pathway has the potential to lead to cancer. The canonical *Wnt* pathway has been implicated in initiation and progression of tumors (Polakis, 2000; Dimitriadis, et al., 2001).

The canonical pathway is a complex cascade of events that ends in the translocation of β-catenin into the nucleus and its binding to the LEF/TCF complex, which then initiates transcription of *Wnt*-target genes. The cascade is initiated when one of the many secreted *Wnt* glycoproteins binds to a Frizzled transmembrane receptor. There are 16 recognized *Wnt* glycoproteins, with many of them partially tissue-specific (Polakis, 2000; Huelsken, 2002).

Ligation of Frizzled (Fz) causes Axin to bind to and phosphorylate Dishevelled (DSH). These two proteins bind to GSK3-β, which, in an unbound state is able to bind to β-catenin and signal it for degradation. This binding thus allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus (Polakis, 2000; Huelsken, 2002). Once in the nucleus, β-catenin binds to the LEF/TCF complex and transcribes many target genes (Cadigan and Nusse, 1997; Cadigan and Liu, 2005). These targets include *Axin 2*, *C-myc*, and *TFRC*, which function in assisting to stabilize β-catenin, regulating chromatin structure, and in the uptake of cellular iron, respectively. Each of these target genes is required for cell proliferation and differentiation and their expression will further enhance the rate of proliferation of cells (Leung, et al., 2002; Rohrs, et al., 2009). This becomes a positive feedback loop with target gene expression allowing cells
to build up and those cells to accumulate β-catenin in the cytoplasm, which results in a nuclear translocation and those target genes being transcribed.

Figure 2. Wnt signaling pathway in an inactive (off) and active (on) states (Jamieson, 2012).

In the non-canonical pathway, β-catenin does not accumulate in the nucleus because it is degraded in the cytoplasm. In this cascade, Wnt proteins do not bind to the Frizzled receptors. This lack of binding keeps Dishevelled from being phosphorylated which allows it, as well as Axin and APC, to bind to β-catenin. The APC/β-catenin complex then recruits GSK3-β to bind and phosphorylate β-catenin (Holcombe, et al., 2002; Reya and Clevers, 2005). Proteasomes carry out the actual degradation process (Leung, et al., 2002). Because of this phosphorylation, β-catenin is degraded and cytoplasmic accumulation is prevented. Subsequently, Wnt target genes are not transcribed and cells are not stimulated to divide, due to lack of a positive growth signal (Dienz and Clevers, 2000; de Sousa, et al., 2011).
In the colorectal epithelial model, the Wnt signal acts on the stem cell populations of the crypts. The signal instructs the stem cells to divide and maintains proliferation. The daughter cells migrate up the crypt wall, pushing the older cells to the top of the crypt where they are sloughed into the lumen as they become effete. The Wnt signal only acts on deep crypt populations, which comprise the stem cells and a few progenitor cells (Kosinski, et al., 2007; Hirata, et al., 2013). In a canonical or de-regulated state, the deep crypt cells continue to proliferate, continually moving up the crypt wall causing cells to build up. Two important proteins in this pathway are APC and β-catenin. These two proteins interact in the normal cells, with APC controlling how often cells divide and how the cell attaches to other cells within a tissue and β-catenin regulating the cell-to-cell adhesion and gene transcription. The genes encoding these two proteins contain the majority of the mutations that result in de-regulation of epithelial growth (Morin, et al., 1997; Schneikert and Behrens, 2007).

**β-Catenin**

β-catenin, coded by the \textit{CTNNB1} gene, is a cadherin-binding protein as well as a transcriptional activator when joined with TCF (Morin, et al., 1997; Feng Han, et al., 2006). β-catenin has important roles in cell adhesion and signal transduction (Huber and Weis, 2001). It stimulates proliferation through modulation of the expression of certain target genes. β-catenin has an amino terminal domain of 149 amino acids with a central domain of 515 amino acids counting 12 armadillo repeats (20 amino acids each) and finally a C-terminal domain with 108 residues. β-catenin is able to bind to APC via the armadillo repeat domain and has phosphorylation sites on Ser45, 41, 37, and 33 of the N-terminal domain that become available after binding for GSK3-β to phosphorylate (Huber and Weis, 2001; Kohler, et al., 2009). APC may be unable to bind to β-catenin if APC is mutated or if β-catenin is mutated. Both defects
result in a de-regulation of the Wnt signal, causing β-catenin to build up in the nucleus which may lead to cancer (Morin, et al., 1997). This build up in the nucleus is greatest in the epithelial colonic crypts with a decreasing gradient of β-catenin as the cells migrate up the crypt wall due to lack of active signal acting on these cells (Pinto and Clevers, 2005; Kosinski, et al., 2007). While APC mutations are very common in CRC, in tumors without APC mutations, a β-catenin mutation is almost always present and occurs in 15% of all CRC cases and commonly occurs in the central domain causing a deletion of about four of the repeat sequences (Shibata, 1997; Polakis, 1999; Polakis, 2000). It is not the deletions themselves that support the development of CRC, but disruption of the binding sites as APC binds to β-catenin between repeat 3 and 4 causes a deletion of these repeats, resulting in an inability of APC to bind Axin (Polakis, 1999).

Many studies have shown that increased β-catenin expression promotes carcinogenesis in a dose-dependent manner. However, a recent study reports that, paradoxically, abundant over-expression of β-catenin may actually slow proliferation of the colonic crypt epithelium, as these cells fail to express Ki-67, a nuclear protein needed for proliferation. This loss of Ki-67, in a mechanism still unknown, prevents proliferation of cells containing high levels of β-catenin and, under the conditions of an initiated cell, would slow any progression by the crypt population toward CRC (Hirata, et al., 2013).

Adenomatous Polyposis Coli (APC)

APC is a tumor suppressor gene, located on 5q2, which acts as a downstream regulator of the Wnt pathway. It regulates cellular levels of β-catenin by targeting it for degradation via the ubiquitin pathway (Dimitriadis, et al., 2001; Kohler, et al., 2009). APC has binding sites for Axin and β-catenin with a segment located between the 20R2 and 20R3 (20 amino acid repeat 2 and 3 respectively), which spans amino acids 1404-1466, being most important for degradation
of β-catenin (Segditsas and Tomlinson, 2006; Kohler, et al., 2009). A mutation in this region would result in the inability to degrade β-catenin. APC also plays roles in chromosomal segregation and any defects would thus promote cancer progression (Schneikert and Behrens, 2007).

Approximately 85% of all colorectal cancers, both sporadic and hereditary, show an inability of APC to function and such tumors also have high levels of β-catenin and loss of APC-mediated regulation (Shibata, 1997; Dienz and Clevers, 2000). Familial adenomatous polyposis (FAP) is a hereditary colorectal cancer that is characterized by large numbers of colon polyps that arise because patients are defective in one APC allele (Reya and Clevers, 2005). Once the second allele becomes defective, cancerous lesions start to form due to complete lack of APC being able to regulate CRC via the Wnt pathway.

Transferrin Receptor Protein (TFRC)

TFRC is a target of the Wnt signaling pathway required for iron delivery from transferrin to cells. TFRC protein is necessary for proliferation as cell growth cannot occur without iron. Because of this, TFRC mRNA expression is increased when cell proliferation is increased, in order to accept more iron from transferrin to satisfy growth needs, and is expressed in high quantities in proliferating or cancerous cells (Ryschich, et al., 2004; Rohrs, et al., 2009).

Axin2

The protein, Axin 2, functions to organize a complex between APC, β-catenin, GSK3-β and conductin. This complex then is able to promote the phosphorylation of β-catenin which marks it for degradation. Without this complex and the ability of Axin 2 to bind to these multiple proteins, β-catenin would not be able to be regulated (Jho, et al., 2002; Leung, et al., 2002). This negative feedback pathway is then able to regulate the Wnt pathway. In this way,
Axin 2 functions as a tumor suppressor as it negatively regulates this growth stimulatory pathway (Polakis, 2000).

**c-Myc**

*c-Myc* is a target of the *Wnt* signaling pathway and is itself a transcription factor, meaning that it can then initiate transcription of other genes that are indirect targets of the *Wnt* pathway. Up-regulation of c-Myc results in cellular proliferation or activation (Rohrs, et al., 2009). c-Myc in some instances acts as a repressor by recruiting histone deacetylases (Rohrs, et al., 2009).

**Wnt 8a**

All Wnt proteins are ligands that bind with certain affinities to any one of the seven Frizzled receptor proteins. *Wnt 8a* has been shown to demonstrate expression differences in normal compared to tumor colonic tissue (Bouillet, et al., 1996).

**Wnt Inducible signaling pathway protein 1 (WISP-1)**

The WISP-1 protein is another target of the *Wnt* signaling pathway. Its expression is greatly increased in neoplastic cells and promotes tumor growth by inducing morphological transformation and cellular saturation (Xu, et al., 2000).

**Secreted Frizzled-Related Protein 4 (SFRP4)**

SFRP4 is a secreted protein that is able to bind to both the Frizzled receptor and *Wnt* ligand via the N-terminal cysteine-rich domain. This binding prevents the Wnt ligand from binding to the receptor protein and blocks the signal from continuing along the pathway (Feng Han, et al., 2006). Colorectal tumors commonly display a hypermethylated promoter region of the *SFRP4* gene which is enough to silence the gene and prevent any suppression of the *Wnt* pathway (Feng Han, et al., 2006). Expression of SFRP4 in colorectal cancer is controversial as some researchers have found no differences between expression in normal and carcinogenic
colonic tissue while others have found significant differences between these two states (Suzuki, et al., 2004; Feng Han, et al., 2006; Huang, et al., 2010).

Understanding the function of each of these Wnt pathway proteins, in both normal and initiated states, is necessary when investigating how to regulate and maintain a normal non-initiated state.

Microbiota of the Colon

The colonic microbiota, in response to changes in the environment, including diet, greatly affects proliferation and differentiation of the colorectal mucosa through the production of various metabolically active compounds. The human microbiota of the colon is home to 10 times as many bacterial cells than there are human cells in the body. Many other organisms, such as archaea, viruses, parasites, and/or fungi also call the human gut home (Moschen, et al., 2012). The microbiota contains over 1,000 distinct species of bacteria (Simon and Gorbach, 1984; Qin, et al., 2010). Many of these species are anaerobic and together have 100- to 200-fold more genes than the human genome (Cummings and Macfarlane, 1997; Ashida, et al., 2012; Moschen, et al., 2012). The colonic microbiota is able to break down carbohydrates normally excreted by the host into short-chain fatty acids (SCFAs), gases, and organic acids. These SCFAs are absorbed and used by many human tissues, such as epithelial cells of the colon and liver, as energy sources (Cummings and Macfarlane, 1997; Jacobasch, et al., 1999). Through this "salvage pathway", the colonic microbiota can have large impacts on host health (Moschen, et al., 2012). Based on the products of fermentation, some genera of the colonic microflora, Bifidobacterium and Lactobacterium, for instance, have health-promoting properties, while others, such as Bacteroides and Clostridium, have health-damaging properties (Wollowski, et al., 2001;
Oberreuther-Moschner, et al., 2004; Lim, et al., 2005). Harmful effects include diarrhea, infections, and carcinogenesis, while health-promoting effects include increased immune function, improved digestion and absorption of nutrients, and synthesis of vitamins (Gibson and Roberfroid, 1995; Grabitske and Slavin, 2009). This large impact of the microbial community on the colon through the salvage pathway, and corresponding end-products of fermentation by individual species, greatly influences colorectal tumorigenesis formation and progression (O'Keefe, et al., 2009).

The intestinal microbial communities are relatively stable within an individual across time, but can be dramatically impacted by the environment, including diet and antibiotic therapy (Gill, et al., 2006; Jalanka-Tuovinen, et al., 2011; Birt and Phillips, 2013). This community is very sensitive to population and environmental changes, as a metabolic end-product of one species may serve as a nutritional source for another. In this way, altering one species may very quickly affect many other species of bacteria (Gibson and Roberfroid, 1995). These bacteria thrive on fermentable carbohydrates and other ingesta that escape small intestine digestion, with bacterial populations changing as food sources change. Altering microbial food sources may provide a means of shifting fermentation products away from those that do not benefit the hosts' health and toward those that do.

**Diet and Lifestyle**

Many studies have centered on the central theme of diet and lifestyle factors significantly impacting the incidence of colorectal cancer. It is not just lifestyle: race and gender also impact responses to various diet components. One study performed in Nashville, Tennessee looked at many factors of lifestyle in relation to colorectal polyp formation in over 6,000 participants (Fu,
et al., 2012). This study found that elevated risk of polyp formation was associated with smoking, obesity, and red meat and alcohol consumption, while reduced risk of polyp development was associated with regular NSAID use and high daily intakes of fiber, vitamin A, and calcium (Wu, et al., 1987; Oberreuther-Moschner, et al., 2004; Fu, et al., 2012). Other studies have shown that various healthy behaviors, such as regular physical exercise and eating large amounts of fruits and vegetables, also relate to a decreased risk of colorectal cancer (Scharlau, et al., 2009). Not all studies have agreed on every aspect of diet and lifestyle in relation to risk of colorectal carcinogenesis. There are, however, two widely accepted colorectal diet associations: dietary fiber is a widely accepted inhibitory factor, while red meat is facilitator of colorectal carcinogenesis.

Dietary fiber has been widely accepted as a prebiotic that is protective against CRC. Many studies, though, have reported varying results about the actual benefits of natural dietary fibers and under what conditions they are active. The recommended intake should be at least 25-30 g/day, with the average human on consuming half that amount. The amount of fiber consumed in each study also highly impacts the resulting conclusions of dietary fiber's effects on CRC, risk with more fiber shown to be more protective (Mai, et al., 2003). Dietary fiber is the indigestible portion of food from the plant and includes many types of resistant starch. Studies agree in general that dose, nature of dietary fiber, and frequency and duration of consumption matter when studying the effects on CRC (Nofrarias, et al., 2007; Mathers, et al., 2012). Findings from a 26-year-long study suggest that fiber from green salads has no effect on risk of CRC, while cooked green vegetables, tomatoes, and legumes have a protective effect (Tantamango, et al., 2011). Grain fibers did not show any statistically significant associations, although participants in this study had a low consumption of eating grains (Tantamango, et al.,
Another large study involving 283,222 women with an average follow up of 8 1/2 years showed no correlation between fiber and CRC. However, the maximum dietary fiber intake in any of the participants was only 16.7 g/day. This low level of fiber intake may be responsible for the no- to low-inverse relationships reported by other studies as it is well below the recommended daily intake. (Schatzkin, et al., 2007).

While dietary fiber consumption has been recommended for CRC prevention, high consumption of red meat has been discouraged (Pierre, et al., 2003; Fu, et al., 2012). As with dietary fiber, many studies report varying results with the amount of meat consumed, preparation method, level of doneness, and frequency of consumption affecting CRC risk. A study conducted in Australia with 1,200 participants aged 40-80 answered a questionnaire on their meat consumption and all aspects of cooking/consuming this meat as well as all other lifestyle factors in relation to polyp formation/removal and CRC occurrence. The study found no significant relation to any meat consumption levels or specific cooking practices to an increased risk of CRC (Tabatabaei, et al., 2011). A meta-analysis of 17 publications on meat consumption in relation to CRC risk showed that an increase of 100g per day of any meat results in a 12-17% increased risk of CRC (Sandhu, et al., 2001). These findings are based on only 17 studies, of which many had co-variables, making it challenging to determine if the effects seen in those studies were due to meat consumption alone or the interactions between variables (Sandhu, et al., 2001). These studies also had many issues with quantifying intake from questionnaires and classifying cooking doneness without actually measuring the doneness of the prepared meat, as perception may vary from one participant to another.

Evaluating effects of diet on CRC has proven challenging, but as more studies are completed, dietary recommendations for prevention of CRC will be strengthened. Diets rich in
meat should be avoided while those with ample amounts of vegetables, fruits, and dietary fibers should be sought after. Physical exercise is recommended while consuming large quantities of alcohol is not. Maintaining a healthy weight also reduces risk of CRC. The amount of food consumed and the way in which it was cooked also matters when evaluating a person's CRC risk (Wu, et al., 1987; Scharlau, et al., 2009).

**Starch**

Starch is the major component of grains and is routinely used in the food industry for cereals and pastas. It is synthesized in the leaves of green plants and used as a storage site for energy (Jane, et al., 1994; Han and Hamaker, 2001). Starch is made up primarily of two glucose polymers, amylose and amylopectin. Amylose is mainly linear $\alpha$-(1, 4)-linked D-glucopyranosyl units with less than 1% glycosidic linkages. It makes up about 15 to 30% of normal starch, but can be up to 85% of some inbred varieties of high amylose corn starch (Tharanathan, 2002). Amylopectin, on the other hand, is extensively branched and contains $\alpha$-(1, 4)-linked D-glucopyranosyl units joined through $\alpha$-(1, 6) linkages (Delcour, et al., 2010). Amylopectin comprises about 20 to 45% of normal starch and has a semi-crystalline structure unlike the amorphous structure of amylose (Tharanathan, 2002). Starches naturally occur as granules, but the dimensions and morphologies vary depending on the plant origin. Corn starch granules typically have a “Maltese cross” appearance when viewed under polarized light (Figure 2) (Baldwin, et al., 1994; Cummings, et al., 1996; Delcour, et al., 2010).

Gelatinization is the process of transforming the starch granules, with an adequate amount of water, into a viscoelastic paste. The process of heating the granules in water causes a swelling of the granule and turns the crystallized structure into a more amorphous form.
(Tharanathan, 2002). Subsequent cooling, called retrogradation, allows the amylopectin and amylose chains to recrystallize (Delcour, et al., 2010). Many factors impact the rate of gelatinization and retrogradation, such as type of plant starch, amylopectin and amylose contents, rate of heating and cooling, and presence of non-starch components (Garcia-Alonso, et al., 1999; Rocha, et al., 2011).

Figure 2. Starch viewed under polarized light showcasing a typical “Maltese cross”.

In order for animals to derive the energy stored in starch, they must be capable of breaking down the α-1, 4 bonds. This is called enzyme hydrolysis and carried out by glucoamylases which are found in saliva and pancreatic secretions of humans and animals and also are secreted by bacteria (Marin-Navarro and Poiaina, 2011).

Resistant starch is starch or a fraction of starch that is not hydrolyzed by enzymes in the digestive tract and is instead available for fermentation by the microflora in the colon (Englyst, 1986; Cummings, et al., 1996). The result of this fermentation is the production of short chain fatty acids (SCFAs), gases, and other organic acids (Grabitske and Slavin, 2009). There are currently five types of resistant starch with classification based on structures and resistant content (Themeier, et al., 2005). Type 1 (RS1) is physically inaccessible to enzymatic
degradation by \( \alpha \)-amylase because it is blocked by the cell wall. The starch granules only become accessible to enough water to fully gelatinize when the cell wall is broken down (Jacobasch, et al., 1999; Themeier, et al., 2005). Resistant starch type 2 (RS2) is a naturally occurring granular starch found in raw potatoes, peas, and green bananas. It has a semicrystalline structure that is able to prevent or to slow enzymatic digestion by \( \alpha \)-amylases (Themeier, et al., 2005; Jiang, et al., 2010). However, when RS2 is cooked, it loses the crystalline structure and becomes completely amorphous, allowing slow, but complete digestion (Tharanathan, 2002; Hasjim, et al., 2010). Type 3 RS (RS3) is retrograded starch resulting from heating and then cooling the starch molecules. This type of resistant starch has a crystalline structure that is able to undergo enzymatic break-down by microbial fermentation, but not by mammalian digestive enzymes (Jacobasch, et al., 1999; Ze, et al., 2012). Starches that can be retrograded to form RS3 have high amylopectin content with chains that are long enough to produce stable crystalline structures (Tharanathan, 2002). Resistant starch type 4 (RS4) is a chemically modified starch that has a decreased accessibility of the enzymes to hydrolyze the granules (Jiang, et al., 2010). RS type 5 (RS5) is a newly developed amylose-lipid complexed starch. This amylose-lipid complex has been shown to resist digestion as the lipids block the chains from being accessed by enzymes (Hasjim, et al., 2010; Jiang, et al., 2010).

The resistance to digestion of resistant starch has many potential health benefits, serving as a prebiotic. It reduces postprandial plasma-glucose and insulin responses, which are components of the pathogenesis of diabetes. Because of lowered blood glucose levels, rates of cholesterogenesis, lipogenesis, and lipolysis are also reduced (Hasjim, et al., 2010; Lattimer and Haub, 2010). Consequently, ingestion of resistant starches is believed to have the ability to reduce the occurrences of cardiovascular diseases and some forms of diabetes. Dietary resistant
starch may also reduce obesity by lowering the total energy intake. Resistant starch stays in the digestive system longer and increases satiety, which may reduce food intake (Raben, et al., 1994). However, resistant starch is a poor source of metabolic energy, which may cause energy to be obtained from other sources, such as other foods, or retrieved from the storage depots in adipose tissue (Raben, et al., 1994).

Resistance starch not only has an effect on the glucose-insulin system, but also has been shown to prevent colon pre-neoplastic lesions in animal models (Clausen, et al., 1991). The SCFAs that are released as byproducts of microbial metabolism have been shown to induce proliferation by cells at the crypt base and to inhibit proliferation at the luminal surface, which allows for tightly controlled epithelial growth (Jacobasch, et al., 1999). Some SCFAs also inhibit DNA synthesis and arrest cell growth in the G1 phase of the cell cycle in neoplastic cells, preventing any further cell growth (Jacobasch, et al., 1999).

Manipulating the variation of SCFAs present in the colon, through host consumption of resistant starch, can provide a highly valuable means to maintain normal colonic cell growth.

**Short Chain Fatty Acids (SCFAs)**

Short chain fatty acids are by-products of the bacterial fermentation process. The pattern of SCFA production is related to the bacterial species composing the microbiome and the composition of the partially digested ingesta that enter the large intestine (Christian, et al., 2003; Duncan, et al., 2004). Acetate, propionate, and butyrate are the primary microbial fermentation by-products found in fecal and cecal samples. Butyrate and propionate have been shown to have beneficial health properties, while acetate has been shown to be the most abundant (Cummings and Macfarlane, 1997; Wolin, et al., 1999). Other SCFAs are present, but are not excreted in the
feces, as they apparently become sources of nutrition for other anaerobic bacteria or are transferred to the host (Duncan, et al., 2004).

Fermentation of plant polysaccharides such as resistant starch is expected to cause an increase in the stool mass and to reduce the transit time of the feces through the digestive system. Both of these factors are shown to reduce the potential exposure of colonocytes to mutagens and carcinogens through dilution (greater mass) and a shorter exposure time (Fung, et al., 2012), thereby reducing DNA damage and the accumulation of cancer-potentiating mutations. Increased SCFA production was also found to decrease luminal pH, which causes a decrease in 7α-dehydroxylase, a bile acid breakdown enzyme. If this enzyme remained active, secondary bile acids are formed that inhibit butyrate from properly regulating cell proliferation (Jacobasch, et al., 1999; Lesmes, et al., 2008).

While acetate and propionate are primarily metabolized by various body tissues, butyrate predominantly stays in the colon, where it is metabolized by the colonic epithelium (Bourquin, et al., 1996). Butyrate is able to induce cell differentiation, promote growth and proliferation of normal tissue of the colon while inhibiting cancerous cell growth (Clausen, et al., 1991). While the other SCFAs leave the colon, butyrate moves to the crypt base where it acts on the epithelial cells, controlling transcription expression and activity of genes and proteins of the apoptotic pathway. Butyrate is able to inhibit DNA synthesis of neoplastic cells by inhibiting histone deacetylase activity, causing them to undergo apoptosis (Nofrarias, et al., 2007; Fung, et al., 2012). Many studies have looked at butyrate and its effects in both animal and human studies with a large amount of those studies demonstrating beneficial properties of butyrate. Increasing concentrations of butyrate in the colonic tissue could possibly provide an excellent approach, for future preventative medications, in inhibiting cancerous cell growth.
Animal Models of Colorectal Carcinoma

Selection of an appropriate animal model is crucial for every study, especially when investigating the initiation and progression mechanisms that underlie the development of CRC. Selection of an appropriate mouse model of CRC is dependent upon the specific mechanism under investigation in order to provide the best laboratory correlate of the human disorder. Genetically modified mouse models like \( APC^{\text{min}+/} \) mice are inappropriate for studying sporadic colon cancer, especially when studying genetic variations caused by diet or lifestyle factors, but are better suited and more commonly used to study hereditary CRC like FAP (Taketo and Edelmann, 2009; De Robertis, et al., 2011). Chemically induced CRC models such as A/J mice are used to studying sporadic CRC (Hung, et al., 2010).

Azoxymethane (AOM) is a commonly used carcinogen for specific induction of colon cancer. Azoxymethane is a metabolite of 1, 2-dimethylhydrazine (DMH) and is a specific colon carcinogen (Hirose, et al., 2003; Bissahoyo, et al., 2005). The carcinogen, AOM, does not interact with DNA directly but is metabolized by cytochrome P450 enzymes, which then allows hydroxylation of the methyl group of AOM (Chen and Huang, 2009; De Robertis, et al., 2011). This hydroxylation results in methylazoxymethanol which breaks down into formaldehyde, a highly reactive alkylating agent, causing alkylation of guanine to O6-methylguanine or O4-methylthymine. The change of guanine to either of these, result in an inappropriate switch of DNA nucleotide pairing from a C:G to A:T with O6-methylguanine binding to thymine and O4-methylthymine binding to adenine. These mutations can occur in any gene, such as \( k-ras \), with many mutations having the capacity to initiate tumorigenesis (Hirose, et al., 2003; Chen and Huang, 2009; Cheung, et al., 2010). The polysaccharide, DSS, is a pro-inflammatory reagent
and commonly used in combination with AOM administration (Tanaka, et al., 2003; Suzuki, et al., 2006; Laroui, et al., 2012). There is a common procedure of using a single AOM injection and DSS in the water for up to 20 weeks to mimic colitis-driven tumor development, but this requires a lengthy study period and results in low tumor multiplicity. (Tanaka, et al., 2003; Suzuki, et al., 2006).

Strains of mice react differently to each of these chemicals, a fact which only further emphasizes the importance of choosing an appropriate mouse model. Selection of a chemically induced mouse model of colon carcinogenesis should be carefully considered. The cell type of interest should respond to the carcinogen of choice without causing death to the rodent. It is also important to recognize the susceptibility to the carcinogen when determining a dosage. When using AOM to induce and mimic a sporadic colorectal carcinogenesis, three mouse strains are commonly used: A/J, SWR/J, and AKR/J. Each of these strains vary in the susceptibility to AOM with A/J being most susceptible with largest number of tumors formed and AKR/J mice showing the fewest tumors develop (Nambiar, et al., 2003; Bissahoyo, et al., 2005). The A/J mouse also had a dose-dependent response to AOM and was also able to tolerate higher doses of AOM than the SWR/J line (Papanikolaou, et al., 1998; Bissahoyo, et al., 2005). The A/J strain was unique among these three strains for the high incidence and range of dysplastic lesions, which serves as a putative surrogate endpoint of CRC, induced by AOM-treatment.

**Overall Summary**

Colorectal cancer is an important cause of mortality in the developed world and occurs as a consequence of mutational loss of regulation of colonic epithelial cell proliferation; with each mutation further stimulating CRC progression. Preventing this loss of regulation may be
possible by manipulating the microbiota, since some metabolic products of microbial fermentation, such as butyrate, are known to regulate epithelial cell proliferation and differentiation through interaction with the Wnt signaling pathway, an important regulator of the colonic epithelium. Delivery of increased fermentation substrates to the colonic microbiota results in increased production of these beneficial substances. Resistant starch is a source of carbohydrate that can be effectively delivered to the colon and used by microbial populations for fermentation, with various resistant starches being differentially fermentable. In this project, I therefore hypothesized that resistant starch and the microbial metabolic products generated from resistant starch have the potential to regulate the Wnt signaling pathway, controlling proliferation of normal and pre-neoplastic (initiated) colonic epithelial cells. To test this hypothesis, I evaluated the effects of several different forms of dietary resistant starch on mRNA levels for genes and downstream targets of the Wnt pathway, an important regulator of epithelial cells proliferation.

References


CHAPTER 2: EFFECTS OF DIETARY RESISTANT STARCH ON THE WNT SIGNALING PATHWAY IN AZOXYMETHANE-DAMAGED RAT COLON

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Nicole Cray\textsuperscript{a,b}, Yinsheng Zhao\textsuperscript{b,c}, Yinan Feng\textsuperscript{d}, Peng Liu\textsuperscript{d}, Linda Pollak\textsuperscript{e}, Susan Duvick\textsuperscript{e}, Diane F. Birt\textsuperscript{b,c}, and Elizabeth M. Whitley\textsuperscript{a,b}

\textsuperscript{a} Department of Veterinary Pathology, College of Veterinary Medicine, 2740 Veterinary Medicine, Iowa State University, Ames, IA, USA 50011-1123
\textsuperscript{b} Interdepartmental Graduate Program in Genetics, 2102 Molecular Biology, Iowa State University, Ames, IA, USA 50011-3260
\textsuperscript{c} Department of Food Science and Human Nutrition, 220 MacKay Hall, Iowa State University, Ames, IA, USA 50011
\textsuperscript{d} Department of Statistics, 2117 Snedecor Hall, Iowa State University, Ames, IA, USA 50011
\textsuperscript{e} Department of Agronomy, 1407 Agronomy Hall, Iowa State University, Ames, IA, USA 50011-1010.
ABSTRACT

Dietary resistant starches (RS) have positive impacts on colon health and have been suggested to reduce colon cancer through production of short chain fatty acids (SCFA) by microbial fermentation. These SCFAs, especially butyrate, are believed to help maintain the health of colonic epithelial cells through unidentified interactions with the apoptotic and Wnt signaling pathways. The Wnt pathway regulates cellular proliferation and differentiation. Aberrant Wnt signaling is common in colorectal cancer, with mis-regulation and over-expression of β-catenin, an intracellular signal transducer of the Wnt signaling pathway, in colonocytes. Given that SCFAs have shown potential benefits on colonic signaling mechanisms, we hypothesized that diets rich in resistant starch will modulate the activity of the Wnt pathway in colonic epithelial cells. We evaluated intestinal mRNA levels of β-catenin and other genes in this pathway among F344 rats fed diets containing 34.5±2.0%, 0.2±0.1%, and 1.9±0.1% resistant starch from corn lines Guat209 (GUAT), AR16035 (AR), or a hybrid (ARxGUAT), respectively, for 8 weeks following initiation of carcinogenesis by azoxymethane (AOM) treatment. Dietary and treatment controls included a diet containing the highly digestible starch, AR, and saline injection, respectively. The incidence of the putative preneoplastic lesions, aberrant crypt foci (ACF), was quantified. Quantitative PCR for genes and targets of the Wnt pathway was performed on full-thickness samples of colon wall and on populations of microdissected epithelial cells of the mucosal surface and crypt base. In physiologically normal (non-AOM-injected control) rats fed GUAT, mRNA for β-catenin in samples from full-thickness colon was lower, compared with samples from rats fed either AR or ARxGUAT. Deep crypt colonocytes in AOM-injected rats fed GUAT displayed a marked and statistically significant decrease in β-catenin mRNA expression, compared with a similar population from the saline-injected rats fed
GUAT, a change not reflected in similarly treated rats fed control diet. These results demonstrate the potential for resistant starch to modulate the \( \textit{Wnt} \) signaling pathway under normal and pre-neoplastic conditions.

**KEYWORDS:**
Adenomatous Polyposis Coli (APC), ARxGUAT, Axin 2, Azoxymethane (AOM), \( \beta \)-catenin, Colorectal cancer, Corn maize, HNFI-\( \alpha \), Laser Capture Microdissection (LCM), Resistant Starch (RS), SFRP4, Transferrin Receptor Protein (TFRC), WISP-1, \( \textit{Wnt} \) pathway

**INTRODUCTION**

Colorectal Cancer (CRC) is the third most commonly diagnosed cancer in both men and women, with approximately 102,480 new cases of colon cancer in the USA in 2013 alone (American Cancer Society, 2013). While the number of people dying from colorectal cancer has slowly begun to decline, probably due to increased screening, current screening methods are inexact and can be expensive and uncomfortable. The use of dietary measures to prevent carcinogenesis and tumor progression holds the possibility of dramatically reducing the incidence of this disease (Clausen, et al., 1991; Lattimer and Haub, 2010).

Dietary resistant starch (RS) shows promise as a prebiotic agent for preventing colorectal cancer by modifying cell proliferation and maturation in colonic crypts, the site of intestinal mucosal renewal and, in the cancer stem cell model, the location of the cell of origin for
colorectal cancer (Vaiopoulos, et al., 2012). Resistant starches are starch molecules or components of starch molecules that are not digested in the small intestine and instead are available for fermentation in the colon (Englyst, 1986). Fermentation by colonic microbes produces short chain fatty acids, including butyrate, which are hypothesized to affect the development of colon cancer by regulating cell proliferation, differentiation, and migration (Fung, et al., 2012).

Two naturally occurring maize (Zea mays) lines were used in this study, AR and GUAT. Hybridization of AR and GUAT lines were conducted, resulting in a hybrid, ARxGUAT. Two different genetic mutations in corn have been shown to result in higher resistant content, starch branching enzyme (sbe1) and amylose extender 1 (ae1). Corn starch from a starch branching enzyme (sbe1) mutant was reported to have an altered molecular structure that resulted in increased resistance to pancreatic α-amylase, β-amylase, and isoamylase, as compared with starch lacking this mutation (Xia, et al., 2011). Amylose extender 1 (ae1) mutants have been shown to have longer internal chain lengths in amylpectin and fewer branched outer chains than a wild-type starch (Liu, et al., 2009). The GUAT line contains mutations in both the sbe1 and ae1 genes.

Butyrate and other products of colonic RS fermentation, through molecular mechanisms not fully understood, are believed to regulate the Wnt signaling pathway in colonic epithelium. This pathway is a critical regulator of normal colonic epithelial maturation, differentiation, and migration, and dysregulation of this pathway is implicated in the development of colorectal cancer (Fung, et al., 2012). The Wnt signaling pathway is active in stem cells and progenitor cells that reside deep within the colonic crypts (Reya and Clevers, 2005). In a cancerous state, the Wnt signaling pathway is activated, with the cascade initiated when one of the 16 recognized
Wnt glycoproteins binds to Frizzled (Fz) or one of the Secreted Frizzled Related Proteins (SFRP) (Polakis, 2000; Huelsken, 2002). Ligation by either Fz or SFRP causes Axin, with the help of APC, to bind to and phosphorylate Dishevelled (DSH). APC and Axin bind to GSK3-β, which, in an unbound state is able to bind to β-catenin and mark it for degradation. This binding of GSK3-β by Axin thus allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus (Polakis, 2000; Huelsken, 2002). Once in the nucleus, β-catenin binds to the LEF/TCF complex and transcribes many target genes (Cadigan and Nusse, 1997; Cadigan and Liu, 2005). These targets include Axin2, WISP1, and TFRC, which function in assisting to stabilize β-catenin, stimulate cell growth, and in the uptake of cellular iron, respectively. Each of these target genes is required for cell proliferation and differentiation and their expression will further enhance the rate of proliferation of cells (Leung, et al., 2002; Rohrs, et al., 2009). This becomes a positive feedback loop, with the help of other transcription factors, such as HNF1α, allowing cells to accumulate β-catenin in the cytoplasm.

In the non-canonical pathway, Wnt proteins do not bind to the Frizzled receptors. This lack of binding allows Axin and APC to bind to β-catenin and recruit GSK3-β to phosphorylate β-catenin (Holcombe, et al., 2002; Reya and Clevers, 2005). Because of this phosphorylation, β-catenin is degraded and cytoplasmic accumulation is prevented. Subsequently, Wnt target genes are not transcribed and cells are not stimulated to divide, due to lack of a positive growth signal (Dienz and Clevers, 2000; de Sousa, et al., 2011).

As the cells migrate up the crypt, there is a progressively diminished effect of Wnt signaling (Nusse, 2008). Since the Wnt pathway is most active in the deep regions of the crypt, the location of the stem cell niche, we hypothesized that dietary resistant starch will inhibit the Wnt signaling pathway of epithelial cells in this important intestinal microenvironment. We also
hypothesize that diets containing the highly resistant starch GUAT will reduce the development of a putative pre-neoplastic lesion, aberrant crypt foci (ACF), in the colonic epithelium of carcinogen-induced rats.

MATERIALS AND METHODS

Maize Hybrids and Starch Preparation. Starch isolated from kernels of two genotypes of maize and a hybrid of those two genotypes was used in this study. One genotype, GUAT209 (also known as GEMS-0067 and labeled GUAT in this study), is derived from a combination of tropical and temperate germplasm and is homozygous for the \textit{amylose extender 1 (ae1)} mutation, which is known to increase levels of resistant starch in the kernel (Campbell et al., 2007). GUAT is a low-yielding Guatemalan landrace line with amylose content up to 70%. In addition to \textit{ae1}, this line contains additional high amylose modifier genes, like \textit{sbe1}, that further increase the level of resistant starch (Wu et al., 2009). The GUAT line is classified as a resistant starch type 2 (RS type 2). The second genotype tested in the study (labeled ARxGUAT) is a hybrid between GUAT and a high-yielding and highly digestible, experimental, exotic Argentinian variety (AR1035 and designated AR in this study) (Table 1). Kernels of the three varieties were produced under similar field conditions, harvested at maturity, dried to approximately 12% moisture, and shelled prior to being used to make the starch for inclusion in dietary treatments described in this study.

Starch Processing. Kernels from the three lines were steeped in a 50-L 0.2\% SO$_2$, 0.5\% Lactic Acid Solution. The kernels were then ground coarsely and the germ separated with a sieve from the fiber slurry, which was further ground finely to allow for separation into
fractions. The fine fiber was separated through a series of washing steps using water, leaving the gluten and starch. The gluten-starch mixture was allowed to settle at a specific gravity of 1.04, resulting in two distinct layers. The starch was then separated from the gluten by the process of tabling (1.5-L/min, 0.6° slope), dried, and ground into a fine powder for use in the study.

**Diets.** Starches from the three maize varieties were cooked by a water-boiling method and then mixed with the remaining components of the diet (Zhao, et al., in press 2014). The diet formulation was based on the American Society for Nutritional Sciences' standard diet recommendations for mature rats (AIN-93M) (Reeves, 1997). However, the 5% cellulose in the AIN-93M was not included and the starch content was increased by this amount, resulting in a 55% starch content (Table 2). The boiling procedure was formulated by Zhao, et al. with fresh diet cooked every other day (Zhao, et al., in press 2014). The resistant starch content of the starches and of freshly made diets were measured by the AOAC method 991.43 (Horwitz, 2003). The water content of these samples was determined by a Sartorius moisture analyzer MA30 (Bohemia, NY). Levels of resistant starch and water content were measured in three randomly selected samples of each starch or diet (Table 3).

**Animals and Housing.** Five-week-old, male, Fischer 344 (F344) rats (Charles River Laboratory, Wilmington, MA) were used for this study. The rats were housed individually in plastic cages with stainless-steel, wire-mesh tops. The rats were divided randomly into three equal diet groups (AR, ARxGUAT, and GUAT) with 15 rats in each group. Once rats were divided into the three feeding groups, stainless-steel wire floors were placed in the cages to prevent coprophagia. The rooms were maintained at 22±1°C with 60±5% humidity and the room
had a 12-hour light-dark cycle. Diet and water were provided *ad libitum*. The animal studies were approved and performed in compliance with the Institutional Animal Care and Use Committee of Iowa State University.

**Carcinogen Treatment.** Azoxymethane (AOM, Midwest Research Institute, Kansas City, MO), a widely accepted colon carcinogen (Bissahoyo, et al., 2005), was injected intraperitoneally (20mg AOM/kg body weight) into 10 rats from each diet group once a week for two weeks. As a negative control, the remaining 5 rats from each diet group received intraperitoneal injections of an equivalent volume of sterile physiological saline at the same time as the administration of AOM.

**Diet Treatment.** All rats were fed the AR diet from arrival until three days after the last AOM injection. The rats in each treatment group were then fed the test or control diets for 10 weeks. Body weights were measured weekly and food disappearance was measured by calculating the difference between the amount of food presented to the animals and the amount remaining uneaten at the end of each feeding. At the end of the 10-week period, rats were humanely euthanized individually by CO$_2$, necropsied immediately, and samples were collected for further analysis.

**Necropsy and tissue sampling.** At necropsy, the liver and cecum, with contents, of each rat was removed and weighed. The colon of each rat was removed, incised longitudinally, and flushed with phosphate buffered saline. Each colon was laid flat and the distal 50 mm segment was cut and fixed flat between two glass slides in 10% formalin for at least 24 hours before staining for
Aberrant Crypt Foci. A 1-cm section of the subterminal region of the colon, 6.5 cm anterior to the anus, was removed and frozen in RNA Later (Life Technologies, Grand Island, NY) until RNA extraction of the full-thickness colon was performed. Additionally, a 3-mm section of the colon, immediately adjacent to the sample used for colonic whole tissue analysis was excised and snap-frozen in O.C.T (Optimal Cutting Temperature) compound, (VWR, Radnor, PA) and stored at -80°C until tissue sections were collected for microdissection.

Cecal Weight and pH. The cecum, after removal, was weighed, and then incised and contents were gently removed and the pH of the contents was measured. The cecal wall was then rinsed in phosphate buffered saline, gently blotted dry with an absorbent paper towel and weighed to record the cecal tissue weight. The cecal content weight was calculated as the difference of the cecal weight and cecal tissue weight.

Aberrant Crypt Foci. The formalin-fixed distal 50 mm section of each colon was stained with 1% alcian blue in acetic acid (pH 2.5), counterstained with 1% neutral red, rinsed, gently blotted dry, placed on a glass microscope slide, and ACF were counted using a light microscope (Bird, 1998).

RNA collection and processing from full-thickness and microdissected colon. Relative mRNA levels for 9 genes were determined from full-thickness tissue samples of the colon, consisting of mucosa, submucosa, muscular, and serosal layers. RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA), further purified with RNeasy Kit (QIAGEN, Valencia,
CA) and the quantity and quality of the isolated RNA was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

For microdissected populations of cells, blocks of tissue containing the 3mm section of colon were equilibrated to -20°C and tissues sectioned to 10 microns, using a cryostat with chuck and cabin maintained at -20°C, and collected onto SuperfrostPlus microscope slides and placed at -80°C until staining and laser microdissection was performed the next day. Sections were stained using the Arcturus HistoGene LCM frozen section staining kit (Life Technologies, Grand Island, NY) and stored in xylene for laser microdissection. Epithelial cell populations from the luminal surface and deep within the crypts, (up to the +3 cells, representing the stem- and progenitor-cell populations), were captured independently with a PixCell II Laser Capture Microdissection System (Life Technologies, Grand Island, NY). Approximately 500 cells were collected for each sample location. Extraction of RNA from each sample was performed according to the PicoPure RNA Isolation protocol (Life Technologies, Grand Island, NY) and residual DNA was removed (DNA nuclease kit, QIAGEN, Valencia, CA). The quantity and quality of the isolated RNA was confirmed with Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) with an average of 8ng/ul and 260/280 ratio of 1.74±0.4. The quality of RNA obtained from the microdissected cells was lower than is usually acceptable for qPRC (1.80), but the amount of RNA obtained from microdissected cells is small, which could contribute to this measurement, and the differences from the accepted quality standard were relatively small.

Semi-qualitative, real-time PCR. DNA-free RNA collected from the colonic tissue specimen was reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). Eight genes
encoding members of the Wnt pathway or a downstream target (APC, β-Catenin, TFRC, Wnt 8a, HNF1A, Axin 2, WISP, and SFRP4 (Table 4) were selected for quantitative real-time PCR analysis (qRT-PCR) to identify effects on regulation of the Wnt signaling pathway among the different treatments (saline vs. AOM) and diets (AR, ARxGUAT, and GUAT). GAPDH was used as a reference gene as our pilot studies demonstrated relatively uniform expression of this gene as compared with PSMB6 and PMMI (data not shown), which was in accordance with previous reports (Rubie, et al., 2005). The primers used for qPCR were designed using the software PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA) and synthesized by Integrated DNA Technologies, Inc. (Table 4). SYBR Green Supermix kit (BioRad, Hercules, CA) was used for the qPCR procedure according to the manufacturer’s instructions. A Roche Light Cycler 96 PCR instrument was used with conditions of 95 °C for 3 min, and 40 cycles of 95 °C for 15 s, annealing temperature of the primer for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min, and 55 °C for 1 min.

DNA-free RNA samples isolated from cells microdissected from the deep regions of the colonic crypts or the luminal epithelial surface of each rat were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). Two genes involved in Wnt signaling, β-Catenin and TFRC, were selected for quantitative real-time PCR analysis (qPCR) with GAPDH used as a reference gene (Table 4). The qPCR procedure was performed using the SYBR Green Supermix kit (BioRad, Hercules, CA) according to the manufacturer’s instructions. A GeneAmp 5700 Sequence Detection System was used with conditions of 95 °C for 3 min, and 45 cycles of 95 °C for 15 s, annealing temperature of the primer for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min, and 55 °C for 1 min.
**Statistical Analysis.** Body weight, food disappearance, liver weight, cecal weights and pH, numbers of ACFs, and average crypts/focus, were analyzed using One-way ANOVA or Kruskal-Wallis tests, depending on normality of the data set. Two-sided t-tests were also applied to test the significant differences between groups. \( \Delta C_q \) values from PCR data from each experiment were calculated (Bustin, et al., 2009). \( \Delta \Delta C_q \) values used to calculate fold-change as described by Pfaffl et al. (Tables 5 and 6). Differences in \( C_q \) values were analyzed using ANOVA with post-test (Two-way analysis of variance and Tukey’s multiple comparison test for normally distributed data or Kruskal-Wallis test and Dunn’s multiple comparison test for data that was not normally distributed) (Tables 7 and 8) and Student's t-tests. All values were reported as mean ± standard deviation, with the exception of fold-change data. A p-value < 0.1 was considered significant for qPCR data while a p-value <0.5 was used for all other data. Data analysis was performed using SAS and/or GraphPad Prism.

**RESULTS**

**Resistant starch content isolated from corn lines and in experimental diets.** The degree of dietary resistance of starch derived from each of the three corn lines, AR, ARxGUAT, and GUAT, are 0.2 ± 0.1%, 1.9 ± 0.1%, and 34.5 ± 2.0%, respectively. Resistant starch content of the AR, ARxGUAT, and GUAT diets as fed were 5.4 ± 0.5%, 8.6 ± 0.6% and 18.6 ± 0.9%, respectively, based on dry weight, and the water content of the diets was 20.0 ± 0.9%, 13.0 ± 1.0%, and 14.1 ± 0.9%, respectively (Table 3).

**Body weight, food disappearance, and liver weight.** Significant differences were not observed in the body weight gain or food disappearance among groups over the 8-week experimental
period. An average of 175 ± 6 g body weight was gained, while there was an average daily food disappearance of 15.2 ± 1.4 g/day. AOM or diet effects were not observed on the liver weight data.

Cecal pH and weights. The pH of the cecal contents of GUAT-fed rats was significantly decreased when compared with cecal pH of rats fed diets containing either AR or ARxGUAT, regardless of the absence or presence of AOM treatment (p<0.0001) (Figure 1A).

Rats fed a diet containing the GUAT starch had significantly elevated cecal tissue weight, when compared with cecal weights of rats in either the AOM- or saline treated groups fed AR or ARxGUAT diets (p<0.0001) (Figure 1C). Cecal content weight was also significantly elevated in rats fed the GUAT diet compared with rats fed AR or ARxGUAT diets in AOM-treated and saline-treated groups (p<0.0002) (Figure 1B).

Aberrant Crypt Foci. Aberrant crypt foci (ACF) were only observed in the colonic mucosae of AOM-treated rats (p<0.0001) (Figure 2A). AOM-treated, GUAT-fed rats had a 37% decrease in ACF number when compared with the AOM-treated, AR-fed rats, but this difference was not statistically significant (p=0.2961). A 26% decrease in ACF number was also present in the ARxGUAT-fed rats compared with the AR-fed rats, also not statistically significant (p=0.4243). The colons of GUAT-fed rats had a statistically significant increase in the average number of crypts/aberrant crypt focus when compared with colons of rats fed the AR diet (p=0.0304) (Figure 2B).
Expression of *Wnt* pathway-related genes in whole tissue specimens of colon. This first experiment used the full-thickness sample of colon, deriving the mRNA from cell populations comprising all of the layers of the colonic wall, mucosa, submucosa, muscular tunics, and serosa. β-catenin mRNA expression was significantly decreased in colonic walls of ARxGUAT-fed rats injected with AOM compared with the ARxGUAT-fed rats injected with saline (p=0.02). β-catenin mRNA expression was also decreased significantly, compared with the AR-fed rats injected with AOM (p=0.05) (Figure 3A). *TFRC* mRNA levels were decreased in the AR-fed rats injected with AOM compared with the AR-fed rats injected with saline (Figure 3B). In rats with saline treatment, the mRNA levels for *TFRC* were lower in ARxGUAT-fed rats compared with AR-fed rats (p=0.04) (Figure 3B). *Axin2* mRNA expression displayed a decreased expression in both the saline and AOM treatment groups of GUAT-fed rats compared with the respective treatment groups fed the AR-diet (p=0.09 and p=0.04, respectively). *Axin2* mRNA expression was also decreased in the AOM-injected rats fed the GUAT diet relative to the AOM-injected rats fed the ARxGUAT diet (p=0.01) (Figure 3C). *HNFIα* mRNA expression was decreased in the AOM-injected rats fed the ARxGUAT diet, compared with both the ARxGUAT-fed rats injected with saline and the AR-fed rats injected with AOM (p=0.08 and p=0.06) (Figure 3D). The mRNA level of *SFRP4* was significantly increased in the GUAT diet group compared with both the AR and ARxGUAT diet groups in AOM-injected rats (p=0.005 and p=0.002, respectively). *SFRP4* mRNA expression was also decreased in saline-injected rats fed either the ARxGUAT or GUAT diets, compared with the AR-fed rats (p=0.06 and p=0.008, respectively) (Figure 3E). *WISP1* mRNA expression in saline-treated rats was lower in ARxGUAT- and GUAT-fed rats compared with AR-fed rats (p=0.04 and p=0.1). Among the AOM-injected rats, rats fed the ARxGUAT diet had decreased mRNA expression of *WISP1*.
compared with rats fed either the AR or GUAT diets (p=0.03 and p=0.06) (Figure 3F). There were no significant differences in ΔCq results for mRNA encoding APC or Wnt8a when comparing AOM-injected rats with saline-injected rats or when comparing data from non-initiated rat colons between diet groups (Figure 3G and H).

**Expression of Wnt pathway genes by surface and crypt colonocytes.** To investigate the effects of dietary RS on mRNA encoding β-catenin or TFRC at the anatomic location of epithelial proliferation and, cells residing in two microenvironmental niches of the epithelium, namely the deep regions of crypts and the luminal surface, sites of stem/progenitor and differentiated cell populations, respectively, were isolated by microdissection and gene expression was evaluated by qRT-PCR. In saline-treated mice fed the GUAT diet, β-catenin mRNA expression was increased compared with the saline-treated mice fed the ARxGUAT diet (p=0.003) (Figure 4B). β-catenin mRNA levels were decreased in deep crypt colonocytes of AOM-injected rats fed the GUAT diet, relative to the saline-injected (control) rats fed the GUAT diet (p=0.03). This significant decrease in β-catenin mRNA level was only present in GUAT-fed rats, and not in either AR- or ARGUAT-fed, AOM-injected rats (Figure 4A and B). β-catenin mRNA expression by surface colonocytes, but not deep crypt cells, was lower in the AOM-injected, ARxGUAT-fed mice relative to the AOM-injected, AR-fed mice (p=0.007). β-catenin mRNA expression, of both the surface and crypt, were both similarly decreased in GUAT-fed rats and not in AR-fed, AOM-injected rats (p=0.09 and p=0.07, respectively). Significant differences were not present in ΔCq data for TFRC expression (Figure 4C and D).
DISCUSSION

The inclusion of highly resistant starch, GUAT, in the diet of rats in the preneoplastic stage of colon cancer resulted in a significant decrease in $\beta$-catenin mRNA levels in the colonic epithelium. A large body of evidence demonstrates that decreased $\beta$-catenin mRNA levels are indicative of a non-active $Wnt$ signaling pathway (Polakis, 1999; Polakis, 2000; Hirose, et al., 2003; Feng Han, et al., 2006; Segditsas and Tomlinson, 2006). This model correlates to our mRNA expression data of other key genes in the $Wnt$ signaling pathway, which also point toward a potentially inactive state of the $Wnt$ signaling pathway in GUAT-fed rats injected with AOM.

Analyses of the starches and diets demonstrate a statistically higher percentage of digestion resistance in starch from the GUAT maize line, compared with starch digestion resistance from the AR or ARxGUAT lines (p<0.05). The rats fed the GUAT diet had significantly increased cecal tissue and content weight compared with rats fed either the AR or ARxGUAT diet, suggesting lower digestibility in the small intestine, with a higher volume of residual starch in the ingesta entering the cecum and colon. The GUAT-fed rats may therefore be experiencing increased colonic fermentation, which we hypothesize to be due to increased amounts of starch available for the colonic microbiota. These increases in weight are hypothesized to be primarily due to an increase in the mass of the food product and a corresponding hypothesized increase in bacterial mass (Konishi, et al., 1984; Wollowski, et al., 2001).

Decreased cecal pH was only present in rats fed the diet containing the highest concentration of resistant starch, GUAT, compared with rats fed AR or ARxGUAT diets, which have a normal pH, regardless of AOM or saline injection status. Microbial fermentation of resistant starch in the rat colon has been shown to not only increase cecal weight, but also
decrease cecal pH (Konishi, et al., 1984). We hypothesize that the lowered pH of the cecal contents reflects increased microbial production of acidic metabolic products, including short chain fatty acids such as butyrate. Future studies are needed to better characterize the microbial responses to dietary resistant starch.

Altered crypt foci, putative preneoplastic lesions, were only observed in rats that had received AOM treatment, which supports the well-established concept of azoxymethane being able to initiate carcinogenesis in the colon (Bissahoy, et al., 2005). The highest number of ACF were measured in the rats fed the diet with highly digestible starch, while the rats fed high levels of resistant starch had the lowest average number ACF; though this difference was not statistically significant (p=0.29). However, GUAT-fed rats injected with AOM had a statistically higher number of ACF multiplicity compared with both the AR- and ARxGUAT-fed mice (p<0.05), indicating the possibility of an interaction between the GUAT diet and AOM treatment that affects differentiation of crypt colonocytes. This increased multiplicity of ACF has been related to a later stage of progression, although recent research suggests that ACF, in general, may not be a highly predictive biomarker for future colon carcinogenesis (McLellan, et al., 1991; Hirose, et al., 2003).

The use of azoxymethane (AOM) has been shown to initiate the Wnt signaling pathway and cause an increase in cell proliferation (Takahashi and Wakabayashi, 2004). In our study, we observed changes in gene expression in AOM-treated rats that were consistent with a mixed pattern of up- and down-regulation of the Wnt pathway. Full-thickness colon samples from rats fed the control diet demonstrate increased Axin2 mRNA in the AOM-initiated mice relative to the saline-injected mice (p=0.12). However, TFRC, SFRP4, and WISP-1 mRNA expression in the full-thickness samples are decreased in AOM-initiated mice relative to the saline-injected
mice fed the control AR diet (p=0.1, p=0.005, and p=0.0003, respectively). An alternate pathway, such as the apoptotic or Notch pathway, may be affecting the mRNA expression of these three genes and potentially other untested genes of the Wnt signaling pathway in the AOM-initiated full-thickness samples of the control fed rats as TFRC, SFRP4, and WISP-1 have been shown to be up-regulated by AOM induction (Chen and Huang, 2009; Davies, et al., 2010; Vaiopoulos, et al., 2012).

Diets containing moderate or high levels of resistant starch also modify mRNA expression of genes of the Wnt signaling pathway. ARxGUAT diet fed to saline-injected mice decreased TFRC, SFRP4, and WISP-1 mRNA levels relative to the control AR fed mice injected with saline (p=0.04, p=0.06, and p=0.04, respectively). Although these three genes play roles in multiple pathways (Rohrs, et al., 2009; Davies, et al., 2010), the ability of the ARxGUAT diet to decrease mRNA expression is still important. ARxGUAT fed in the diet not only reduces mRNA expression in saline-injected mice, but also in those injected with AOM. β-catenin, HNF1α, SFRP4, and WISP1 mRNA levels are all decreased in the full-thickness colonic tissue in AOM-injected rats fed the ARxGUAT diet, relative to the control, AR-fed mice injected with AOM (p=0.05, p=0.06, p=0.08, and p=0.03, respectively), suggesting that ARxGUAT fed in the diet may be able to down-regulate the Wnt signaling pathway in an initiated-state. This study only looked at the preneoplastic stage of colon cancer, but the ability of a moderately resistant starch, ARxGUAT, in the diet to decrease mRNA expression levels at this stage demonstrate that resistant starch has strong potential for inhibiting cell proliferation.

A highly resistant starch, GUAT, in the diet has also demonstrated an ability to effectively decrease mRNA expression in the full-thickness colon samples and in site specific, (surface or crypt) epithelial cell populations of the colon. Mice fed the GUAT diet and injected
with saline have decreased *TFRC, Axin2, SFRP4*, and *WISP-1* full-thickness mRNA levels relative to the control, AR-fed mice injected with saline (p=0.10, p=0.09, p=0.008, and p=0.10, respectively). These mRNA expression levels demonstrate that both low and high levels of resistant starch content in the diet are able to down-regulate the *Wnt* signaling pathway. GUAT in the diet is able to decrease mRNA expression of *Axin2* in the full-thickness colon, which was not down-regulated by the ARxGUAT diet. The decrease of another gene is hypothesized to be due to the increased resistant starch content of GUAT and suggests that a higher level of resistant starch has a greater impact on maintaining the colon in a normal, non-initiated, state. GUAT fed in the diet does not only reduce mRNA expression of the full-thickness colon in saline-injected mice, but also in those injected with AOM. The *Axin2* mRNA level of the full-thickness colon is decreased in AOM-injected rats fed the GUAT diet relative to the control, AR, fed rats injected with AOM (p=0.04). The decrease in *Axin2* mRNA expression suggests an ability of this dietary resistant starch, GUAT, to not only influence expression in a saline-injected model, but also in an AOM-injected model. GUAT fed in the diet of rats injected with AOM also had a decreased β- catenin mRNA expression both the colonic epithelial surface and epithelial crypt relative to AOM-injected rats fed the AR, control, diet (p=0.007 and p=0.07). β-catenin mRNA levels are also decreased in the AOM-injected rats fed the GUAT diet relative to the saline-injected rats fed the GUAT diet (p=0.03). Decreased β-catenin levels have been shown to be indicative of a non-active *Wnt* signaling pathway. This decrease in β-catenin mRNA levels of the full-thickness tissue and of the niche specific cells correlates to our mRNA expression data of other key genes in the *Wnt* signaling pathway, which also point toward a potentially inactive state of the *Wnt* signaling pathway in GUAT-fed rats injected with AOM.
Overall, these results demonstrate that increased levels of dietary resistant starch were able to modify the expression of genes encoding $\beta$-catenin, TFRC, Axin2, SFRP4, and WISP-1 of the Wnt signaling pathway in both the full-thickness colon and in the stem cell niche and mature surface colonocytes of rats injected with AOM and fed the ARxGUAT or GUAT diet. The GUAT diet was able to decrease the mRNA expression of a few more genes than that of the ARxGUAT diet in AOM-injected rats suggesting that the level of resistant content is important and that a higher resistant content may be able to decrease overall Wnt signaling to a greater degree.

With the exception of the increased ACF multiplicity, mRNA data suggest an ability of GUAT fed in the diet to inhibit the Wnt signaling pathway. This suggests an ability of resistant starch to decrease the mRNA expression of key genes of the Wnt signaling pathway (Giles, et al., 2003; Segditsas and Tomlinson, 2006). Future studies are needed to investigate the effects of both AOM and diet on the protein levels of the Wnt signaling pathway.

We also demonstrated the important differences in gene expression patterns between samples derived from whole tissue and discrete populations of cells residing in different mucosal microenvironments. This highlights the importance of evaluating mRNA levels from specific populations of cells.

**ABBREVIATIONS**

ACF, Aberrant Crypt Foci

AOM, Azoxymethane

AR, AR starch diet

ARxGUAT, Hybrid resistant starch diet from parental lines, AR and GUAT
CRC, Colorectal cancer

GUAT, Guat starch diet

LCM, Laser Capture Microdissection

O.C.T., Optimal Cutting Temperature

RS, Resistant Starch

SAFETY

Azoxymethane is a potent carcinogen used experimentally to induce colon cancer in rats and mice. It might cause harm to human if swallowed, inhaled or absorbed through skin.

ACKNOWLEDGEMENTS

This research was funded by the Plant Sciences Institute at Iowa State University and a United States Department of Agriculture grant administered through the Nutrition and Wellness Research Center at ISU. We appreciate the contributions of Elise Huffman and Esther Haugabrooks for assisting with collection of samples, Toni Christofferson and Jenni Groeltz for histopathology processing and sectioning, Margie Carter for assistance with LCM, and to Dr. Angela Pillatzki for assisting in optimizing research protocols. We include a very special thank you to Dana Pralle, Carter Roberts, Stephanie Nicholson, and Hannah Rupp for assistance with processing tissue samples and RNA isolation.

REFERENCES


FIGURE LEGENDS

Figure 1. Cecal endpoints from AOM-initiated experimental and saline-injected control rats fed diets containing AR, ARxGUAT, or GUAT starches: (A) pH of cecal contents; (B) Cecal content weight (g); (C) Weight of cecal wall (g). Values are means ± SD. Mean values are significantly different from those for the both the AR and ARxGUAT Saline group (*bars) or both the AR and ARxGUAT AOM group (**bars) with both representing P<0.05.

Figure 2. Altered crypt foci observed in the distal colon of AOM-initiated rats fed diets containing AR, ARxGUAT, or GUAT starches (rats were euthanized 9 weeks after the first AOM injection): (A) average ACF number; (B) average crypts/focus in ACF. Values are means ± SD. *bars are significantly different P-value < 0.05 with n=10 for all AOM-injected groups and n=2-3 for saline-injected control groups.

Figure 3A-H. mRNA expression of Wnt pathway-related genes in the full thickness colon of rats fed diets containing AR, ARxGUAT, or GUAT starches. Data reported are ∆Cq values, using the ΔΔCq method. Each AOM group had n=10 while each saline group had n=2-3. Bars represent statistical significance with one star (*) representing= p-value <0.1, two (***) represent= p<0.05, and three (****) represent= p<0.001. P-values of the Two-Way ANOVA analysis for each mRNA species are listed in Table 7.

Figure 4A-D. mRNA expression measured as ∆Cq of β-Catenin and TFRC standardized using the ΔΔCq Method for surface and crypt niches. Each AOM group had n=10 while each saline group had n=2-3. Bars represent statistical significance with one star (*) representing= p-value <0.1, two (**) represent= p<0.05, and three (****) represent= p<0.001. P-values of the Two-Way ANOVA analysis for β-Catenin and TFRC mRNA are listed in Table 8.
FIGURES
Figure 1

A  pH of Cecal Contents

B  Average Weight of Cecal Content

C  Average Weights of Cecal Tissue
Figure 2

A. Average ACF

B. Multiplicity of Crypts in Altered Crypt Foci
Figure 3 Continued

C'  

Axin 2

D

HNF1A

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**Significance**:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
Figure 3 Continued

E. SFRP4

F. WISP
Figure 3 Continued

**G**

![Diagram showing Delta Cq values for different conditions: AR Saline, AR AOM, ARxGUAT Saline, ARxGUAT AOM, GUAT Saline, GUAT AOM.]

**H**

![Diagram showing Delta Cq values for different conditions: AR Saline, AR AOM, ARxGUAT Saline, ARxGUAT AOM, GUAT Saline, GUAT AOM.]

**Wnt 8a**
Figure 4

A

**β-catenin expression of Surface**

![Graph A]

B

**β-catenin expression of the Crypt**

![Graph B]
Figure 4 Continued

C

TFRC mRNA at Surface

D

TFRC mRNA in Crypts
TABLE LEGENDS

Table 1. Pedigree Information and Nomenclature for Corn Lines.

Table 2. Diet Ingredients. Components of the starch diet as characterized by Zhao et al (in press 2014).

Table 3. Resistant Starch and Water Content of Starches and Diets. Percentage resistant starch content of the starch and of the diet was determined using the AOAC method 991.43.

Table 4. Primer Sequences for qPCR of Rat Colon Samples.

Table 5. Modulation of mRNA expression of genes of the Wnt pathway by dietary resistant starch in full-thickness colon tissue samples of AOM-injected and control rats. With comparisons made within and across diets ∆∆Cq method. * indicates statistical significance (p<0.10).

Table 6. Modulation of mRNA expression of β-catenin and TFRC in Surface and Crypt Niches. Comparisons made within and across diets using the ∆∆Cq method. * indicates statistical significance (p<0.10).

Table 7. P-values of Two-Way ANOVA of genes of the Wnt pathway in full-thickness colon tissue samples.

Table 8. P-values of Two-Way ANOVA of mRNA expression of β-catenin and TFRC in Surface and Crypt Niches.
### TABLES

#### Table 1. Pedigree information nomenclature for corn lines.

<table>
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<th>Abbreviation</th>
<th>Field Data of Corn Lines</th>
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#### Table 2: Diet Ingredients

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<th>Diet Ingredient</th>
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<td>cellulose (insoluble fiber)</td>
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<td>corn oil</td>
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1. Starches were cooked using a water-boiling method as described in the text. All non-starch diet ingredients were purchased from Harland Teklad (Madison, WI) or Spectrum (Gardena, CA).
Table 3: Resistant starch and water content of starches and diets.

<table>
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<th>Starch Groups</th>
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<th>Resistant Starch Content of the Diets on a Dry Diet Basis (n=3) (% ± sd)</th>
<th>Water Content of the Diets (n=3) (% ± sd)</th>
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<td>AR</td>
<td>0.2 ±0.1%</td>
<td>5.41±0.53%</td>
<td>20.01±0.92%</td>
</tr>
<tr>
<td>ARxGUAT</td>
<td>1.9 ±0.1%</td>
<td>8.57±0.59%</td>
<td>12.99±0.98%</td>
</tr>
<tr>
<td>GUAT</td>
<td>34.5 ±2.0%</td>
<td>18.58±0.90%</td>
<td>14.12±0.89%</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Resistant starch contents of diets were measured by AOAC method 991.43.
Table 4. Primer Sequences for qPCR of Rat Colon Samples

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: ACAGTCGATTACAGACAGGCAGCAATT Reverse: TTCACTGTGTCACGACAGCTTT</td>
</tr>
<tr>
<td>APC</td>
<td>Forward: ACCAATATGCGTTATCAAGCGCGTCGCT Reverse: CGATGCGACACAGGCACAAACAT</td>
</tr>
<tr>
<td>Axin 2</td>
<td>Forward: GAGGATGTCTTTCTGCGGGG Reverse: AGCACTGAGCAACTGAGGTC</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Forward: AGGAAGTGGCTGTCAAGGAGGAAA Reverse: TGGCTTTACTTCTTCCACGGGTGA</td>
</tr>
<tr>
<td>HNF1A</td>
<td>Forward: TAAGGATCCCACCCTGTCCC Reverse: CCCCAGTTACCTTCCCC</td>
</tr>
<tr>
<td>SFRP4</td>
<td>Forward: CTGCTTAGAGGCCACCAGGTGTT Reverse: TGAGAGCGGTTCTTAGCCACC</td>
</tr>
<tr>
<td>TFRC</td>
<td>Forward: CAAGACAGCTCAAACAGTGCT Reverse: GCTGCACAAAGTGCGATG</td>
</tr>
<tr>
<td>WISP-1</td>
<td>Forward: AGAAGGGGTAGTGAGGGAAG Reverse: AGCCGGCATGTTCTTACGTA</td>
</tr>
<tr>
<td>Wnt 8a</td>
<td>Forward: ACAGTGAGGCTACAGGGCTATCAT Reverse: TCCTGACAGGGCATACCAATGCTT</td>
</tr>
</tbody>
</table>
Table 5. Modulation of mRNA expression of genes of the *Wnt* pathway by dietary resistant starch in full-thickness colon tissue samples of AOM-injected and control rats. * indicates statistical significance (p<0.10).

### Effect of Dietary Resistant Starch on Gene Expression in Control, Saline-Treated (normal) Colon (b)

<table>
<thead>
<tr>
<th>Diets (a)</th>
<th>β-catenin</th>
<th>APC</th>
<th>TFRC</th>
<th>Wnt 8a</th>
<th>Axin2</th>
<th>HNF1A</th>
<th>SFRP4</th>
<th>WISP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARGUAT</td>
<td>0.90</td>
<td>1.05</td>
<td>9.98*</td>
<td>1.28</td>
<td>2.70</td>
<td>0.37</td>
<td>12.01*</td>
<td>8.94*</td>
</tr>
<tr>
<td>GUAT</td>
<td>1.04</td>
<td>1.64</td>
<td>1.88*</td>
<td>0.61</td>
<td>12.97*</td>
<td>0.49</td>
<td>6.42*</td>
<td>9.14*</td>
</tr>
</tbody>
</table>

a= animals not treated with AOM; ΔΔCq calculated comparing test diets to control (AR) diet  
b= fold change

### Effect of Dietary Resistant Starch on Gene Expression in AOM-Induced Colon (b)

<table>
<thead>
<tr>
<th>Diets (c)</th>
<th>β-catenin</th>
<th>APC</th>
<th>TFRC</th>
<th>Wnt 8a</th>
<th>Axin2</th>
<th>HNF1A</th>
<th>SFRP4</th>
<th>WISP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARGUAT</td>
<td>2.82*</td>
<td>3.30</td>
<td>1.46</td>
<td>1.88</td>
<td>0.62</td>
<td>5.06*</td>
<td>3.37*</td>
<td>3.17*</td>
</tr>
<tr>
<td>GUAT</td>
<td>2.76</td>
<td>1.80</td>
<td>1.36</td>
<td>3.79</td>
<td>11.29*</td>
<td>1.79</td>
<td>0.03*</td>
<td>0.51</td>
</tr>
</tbody>
</table>

b= fold change  
c= animals treated with AOM; ΔΔCq calculated comparing test diets to control (AR) diet

### Effect of AOM-Induction on Gene Expression in the Colon (b)

<table>
<thead>
<tr>
<th>Treatments (d)</th>
<th>β-catenin</th>
<th>APC</th>
<th>TFRC</th>
<th>Wnt 8a</th>
<th>Axin2</th>
<th>HNF1A</th>
<th>SFRP4</th>
<th>WISP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>1.06</td>
<td>1.26</td>
<td>4.57*</td>
<td>0.64</td>
<td>4.66</td>
<td>0.33</td>
<td>12.43*</td>
<td>8.34*</td>
</tr>
<tr>
<td>ARGUAT</td>
<td>3.31*</td>
<td>3.96</td>
<td>0.67</td>
<td>0.93</td>
<td>1.07</td>
<td>4.54*</td>
<td>3.49</td>
<td>2.95</td>
</tr>
<tr>
<td>GUAT</td>
<td>2.80</td>
<td>1.38</td>
<td>3.31</td>
<td>3.92</td>
<td>4.05</td>
<td>1.21</td>
<td>0.06*</td>
<td>0.46</td>
</tr>
</tbody>
</table>

b= fold change  
d= ΔΔCq calculated comparing AOM-treatment with saline-treatment for each diet
Table 6. Modulation of mRNA expression of β-catenin and TFRC in Surface and Crypt Niches. * indicates statistical significance (p<0.10).

**Effect of Dietary Resistant Starch on gene expression in saline-treated (normal) colon (b)**

<table>
<thead>
<tr>
<th>Diets (a)</th>
<th>Beta-catenin Surface</th>
<th>Beta-catenin Crypt</th>
<th>TFRC Surface</th>
<th>TFRC Crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARxGUAT</td>
<td>3.91</td>
<td>8.16*</td>
<td>0.93</td>
<td>0.40</td>
</tr>
<tr>
<td>GUAT</td>
<td>0.78</td>
<td>0.28</td>
<td>0.43</td>
<td>0.10</td>
</tr>
</tbody>
</table>

a= animals not treated with AOM; ΔΔCq calculated comparing test diets to control (AR) diet  
b= fold change

**Effect of Dietary Resistant Starch on Gene Expression in AOM-Induced colon (b)**

<table>
<thead>
<tr>
<th>Diets (c)</th>
<th>Beta-catenin Surface</th>
<th>Beta-catenin Crypt</th>
<th>TFRC Surface</th>
<th>TFRC Crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARxGUAT</td>
<td>19.27*</td>
<td>2.15</td>
<td>0.96</td>
<td>0.16</td>
</tr>
<tr>
<td>GUAT</td>
<td>7.12*</td>
<td>7.65*</td>
<td>1.11</td>
<td>0.28</td>
</tr>
</tbody>
</table>

b= fold change  
c= animals treated with AOM; ΔΔCq calculated comparing test diets to control (AR) diet

**Effect of diet and AOM-Induction on Gene Expression in the Colon (b)**

<table>
<thead>
<tr>
<th>Treatments (d)</th>
<th>Beta-catenin Surface</th>
<th>Beta-catenin Crypt</th>
<th>TFRC Surface</th>
<th>TFRC Crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>0.55</td>
<td>3.23</td>
<td>0.45</td>
<td>2.84</td>
</tr>
<tr>
<td>ARxGUAT</td>
<td>2.71</td>
<td>0.85</td>
<td>0.47</td>
<td>1.13</td>
</tr>
<tr>
<td>GUAT</td>
<td>5.03</td>
<td>88.53*</td>
<td>1.18</td>
<td>7.58</td>
</tr>
</tbody>
</table>

b=fold change  
d= ΔΔCqs calculated comparing AOM-treatment with saline-treatment for each diet
Table 7. P-values of Two-Way ANOVA of genes of the *Wnt* pathway in full-thickness colon tissue samples.

<table>
<thead>
<tr>
<th></th>
<th>β-catenin</th>
<th>APC</th>
<th>TFRC</th>
<th>Wnt 8a</th>
<th>Axin 2</th>
<th>HNF1α</th>
<th>SFRP4</th>
<th>WISP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.58</td>
<td>0.76</td>
<td>0.57</td>
<td>0.57</td>
<td>0.56</td>
<td>0.09</td>
<td>0.002</td>
<td>0.09</td>
</tr>
<tr>
<td>Diet (a)</td>
<td>0.62</td>
<td>0.77</td>
<td>0.40</td>
<td>0.86</td>
<td>0.007</td>
<td>0.67</td>
<td>0.001</td>
<td>0.04</td>
</tr>
<tr>
<td>Treatment (b)</td>
<td>0.10</td>
<td>0.39</td>
<td>0.36</td>
<td>0.70</td>
<td>0.13</td>
<td>0.78</td>
<td>0.58</td>
<td>0.12</td>
</tr>
</tbody>
</table>

a=p-values of the effect of the diet with p<0.1 being significant  
b=p-values of the effect of either saline or AOM injection with p<0.1 being significant

Table 8. P-values of Two-Way ANOVA of mRNA expression of β-catenin and TFRC in Surface and Crypt Niches.

<table>
<thead>
<tr>
<th></th>
<th>β-catenin Crypt</th>
<th>β-catenin Surface</th>
<th>TFRC Crypt</th>
<th>TFRC Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.07</td>
<td>0.59</td>
<td>0.71</td>
<td>0.90</td>
</tr>
<tr>
<td>Diet (a)</td>
<td>0.30</td>
<td>0.11</td>
<td>0.32</td>
<td>0.94</td>
</tr>
<tr>
<td>Treatment (b)</td>
<td>0.03</td>
<td>0.44</td>
<td>0.27</td>
<td>0.62</td>
</tr>
</tbody>
</table>

a=p-values of the effect of the diet with p<0.1 being significant  
b=p-values of the effect of either saline or AOM injection with p<0.1 being significant
CHAPTER 3: STEARIC ACID-COMPLEXED HIGH-AMYLOSE CORNSTARCH MODULATES THE WNT SIGNALING PATHWAY IN AZOXYMETHANE-INITIATED DEEP CRYPT EPITHELIAL CELLS OF THE MOUSE COLON

Modified from a paper to be submitted to The Journal of Nutrition

Nicole Cray\textsuperscript{a,b}, Bridget Nelson\textsuperscript{c,d}, Yinan Feng\textsuperscript{e}, Peng Liu\textsuperscript{e}, Yongfeng Ai\textsuperscript{d}, Jay-lin Jane\textsuperscript{d}, Diane F Birt\textsuperscript{b,c,d}, Elizabeth M Whitley\textsuperscript{a,b}

\textsuperscript{a} Department of Veterinary Pathology, College of Veterinary Medicine, 2740 Veterinary Medicine, Iowa State University, Ames, IA, USA 50011-1123.
\textsuperscript{b} Interdepartmental Graduate Program in Genetics, 2102 Molecular Biology, Iowa State, University, Ames, IA, USA 50011-3260
\textsuperscript{c} Graduate Program in Toxicology, 2102 Molecular Biology, Iowa State University, Ames, IA, USA 50011-3260
\textsuperscript{d} Department of Food Science and Human Nutrition, 220 MacKay Hall, Iowa State University, Ames, IA, USA 50011-1123
\textsuperscript{e} Department of Statistics, 2117 Snedecor Hall, Iowa State University, Ames, IA, USA 50011
Aberrant Wnt signaling is common in colorectal cancer, with a key feature being mis-regulation and over-expression of β-catenin, an intracellular signal transducer of the Wnt signaling pathway by colonocytes. Resistant starches (RS), the portion of non-digestible starch, have been suggested to reduce colon cancer through production of short chain fatty acids (SCFAs) by the colonic microflora and their fermentation process. Butyrate, a SCFA produced by the microbial fermentation, assists in maintaining the colonic epithelial proliferation through undefined interactions with the apoptotic and Wnt signaling pathways. We hypothesize that an increase in dietary resistant starch will modulate the action of the Wnt pathway in colonic crypt epithelial cells. We evaluated mRNA levels of β-catenin and other key genes in the Wnt pathway in colonic crypt epithelial cells, comparing results from A/J mice fed diets containing novel resistant starches. Diets contained a high-amylose cornstarch (HA7), high-amylose 7 complexed with octenyl-succinic anhydride RS (OS-HA7), high amylose 7 complexed with a stearic acid lipid (SA-HA7), or commercially available corn starch (CS), with 19.0 ±1.8%, 4.7±0.4%, 31.1±1.7%, and 3.7±0.7% digestion resistance, respectively. Carcinogenesis was initiated by 4 weekly IP injects of azoxymethane (AOM) at a 7.5mg/kg dose prior to feeding diets containing resistant starch. Dietary and treatment controls included the commercially available corn starch, CS, and saline injection, respectively. Populations of histologically normal epithelial cells of the crypt base were collected by laser microdissection and quantitative PCR performed for genes and targets of the Wnt pathway. Results demonstrate that a diet containing highly resistant starch results in a normalizing effect on upstream genes of the Wnt signaling pathway, and that resistant starch therefore has the potential to effectively inhibit the activity of the Wnt signaling pathway.
**INTRODUCTION**

Colorectal cancer is the third most commonly diagnosed cancer in both men and women with approximately 102,480 new cases of colon cancer in the USA in 2013 alone (Society, 2013). Altering the microbiome through the use of dietary preventative measures, such as prebiotics and other food sources, have been shown to help maintain normal colonic epithelium and reduce the incidence of this disease (Clausen, et al., 1991; Lattimer and Haub, 2010).

Dietary resistant starch (RS) is starch or parts of starch molecules that are not digested in the small intestine and instead are available for fermentation in the colon. Fermentation by the colonic microbiota produces metabolic products such as short chain fatty acids, including butyrate, which have been shown, through unknown mechanisms, to reduce the development of colon cancer (O'Keefe, et al., 2009; Scharlau, et al., 2009). Dietary resistant starch shows promise as a prebiotic for preventing colorectal cancer by modifying genes needed for proliferation and maturation of cells in colonic crypts, the site of intestinal mucosal renewal (Fung, et al., 2012).

There are currently 5 types of resistant starch, with each type representing a different mechanism of resistance. Type two resistant starch (RS2) is starch that occurs in its natural granular form and commonly contains a high amylose content. This study used HA7 (high-
amylose starch VII, a type 2 RS). Type four resistant starches (RS4) are starches that have been chemically modified to resist digestion and are not found naturally. This study used OS-HA7, a high-amylose cornstarch complexed with octenyl succinic acid and resists digestion through its hydrophobic tail complex. Type five resistant starch (RS5) is a high-amylose cornstarch complexed with a stearic acid to result in an amylose-lipid complex (Hasjim, et al., 2010). This RS5 starch is designated SA-HA7 (high amylose-stea

The Wnt signaling pathway is a critical regulator of normal colonic epithelial maturation and differentiation, with de-regulation of this pathway implicated in the development of colorectal cancer. This pathway may be regulated by butyrate and other products of colonic microbial fermentation of resistant starch, in a molecular mechanism not fully understood (Fung, et al., 2012). The Wnt signaling pathway acts in the stem cells and progenitor cells deep within the colonic epithelial crypts (Reya and Clevers, 2005). In a cancerous state, the Wnt signaling pathway is activated, with the cascade initiated when one of the 16 recognized Wnt glycoproteins binds to Frizzled (Fz) or one of the Secreted Frizzled Related Proteins (SFRP) (Polakis, 2000; Huelsken, 2002). Ligation either Fz or SFRP causes Axin, with the help of APC, to bind to and phosphorylate Dishevelled (DSH). APC and Axin bind to GSK3-β, which, in an unbound state is able to bind to β-catenin and signal it for degradation. This binding of GSK3-β by Axin thus allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus (Polakis, 2000; Huelsken, 2002). Once in the nucleus, β-catenin binds to the LEF/TCF complex
and transcribes many target genes (Cadigan and Nusse, 1997; Cadigan and Liu, 2005). These targets include *Axin1* and *TFRC*, which function in assisting to stabilize β-catenin and in the uptake of cellular iron, respectively. Each of these target genes is required for cell proliferation and differentiation and their expression will further enhance the rate of proliferation of cells (Leung, et al., 2002; Rohrs, et al., 2009). This becomes a positive feedback loop, allowing cells to accumulate β-catenin in the cytoplasm.

In the non-canonical pathway, Wnt proteins do not bind to the Frizzled receptors. This lack of binding allows Axin and APC to bind to β-catenin and recruit GSK3-β to phosphylorate β-catenin (Holcombe, et al., 2002; Reya and Clevers, 2005). Because of this phosphyloration, β-catenin is degraded and cytoplasmic accumulation is prevented. Subsequently, *Wnt* target genes are not transcribed and cells are not stimulated to divide, due to lack of a positive growth signal (Dienz and Clevers, 2000; de Sousa, et al., 2011).

The cells become more differentiated as they migrate up the crypt wall, and they are no longer acted on by Wnt signal as it is too weak to transfer from cell to cell to reach the topmost cells. When they reach the upper portions of the crypts, these terminally differentiated cells are unable to initiate cell proliferation through β-catenin nuclear transduction (Nusse, 2008). Specifically evaluating gene expression in epithelial cells of the colonic crypt, the site of proliferation, is important as the *Wnt* signaling pathway does not act on other cell populations.

We hypothesized that the microbial fermentation products of resistant starch, SCFAs, which have been shown to be beneficial in inhibiting colorectal cancer through an undetermined mechanism, will be able to effectively control the *Wnt* signaling pathway, regulating proliferation of normal and pre-neoplastic (initiated) colonic crypt epithelial cells.
MATERIALS AND METHODS

Diets. This study used three different types of resistant starches, HA7, OS-HA7, and SA-HA7, each from a single processing batch and a readily digestible, commercially available cornstarch (CS) as a control. HA7 is a high amylose 7 cornstarch classified as type two resistant starch, OS-HA7 is a high amylose 7 cornstarch complexed with octenyl succinic acid and is classified as a type four resistant starch, and SA-HA7 (Resistant Starch type 5) is a high amylose 7 cornstarch complexed with stearic-acid (Hasjim, et al., 2010). The four diets, containing HA7, OS-HA7, SA-HA7, and CS starches, were cooked by a water-boiling method and mixed with the remaining components of the diet (Zhao, et al., in press 2014). The diet formulation was based on the American Society for Nutritional Sciences’ standard diet recommendations for mature mice (AIN-93M) (Reeves, 1997). The 5% cellulose in the AIN-93M diet was not included and instead, the starch content was increased an extra 5% for a total of 55% starch content (Table 1). All diets were made fresh and fed every two days while the control diets were fed every other day, to preserve freshness, once the experimental diets were fed. The resistant starch contents of the starches and diets were measured fresh by both the Englyst Method and AOAC Method 991.43 (Englyst, 1986; Horwitz, 2003). The water content of these samples was determined by a Sartorius moisture analyzer MA30 (Bohemia, NY). Both the resistant starch and water contents are listed in Table 2.

Animals and Housing. A total of 60, 5-week-old, male A/J mice were received from Jackson Laboratory (Bar Harbor, Maine). The mice were individually housed in plastic cages with stainless-steel, wire-mesh tops. Additionally, stainless-steel wire floors were also placed in the cages to prevent coprophagia during the time the four experimental diets were fed. The room
was kept at 22±1°C with 60±5% humidity with a 12-hour light cycle. Diet and water were available *ad libitum*. The animal studies were approved by and performed in compliance with the Institutional Animal Care and Use Committee of Iowa State University. The mice were randomly assigned to four diet groups (Control, HA7, OS-HA7, and RS5) with 15 mice in each group.

**Carcinogen Treatment.** Azoxymethane (AOM) was purchased from Midwest Research Institute (Kansas City, MO) and injected intraperitoneally once a week for four weeks into 10 mice from each diet group at an 7.5mg AOM/kg mouse body weight dosage. The remaining 5 mice from each diet group received intraperitoneal injections of physiological saline, as a negative control.

**Diet Treatment.** All mice were placed on a semi-purified control diet from arrival to three days after the last AOM injection. The mice were then divided randomly into their treatment groups and fed the respective diets for 10 weeks. Body weights were measured weekly and food disappearance was measured by calculating the difference between the food presented to each animal and the amount left over at the end of each feeding. Food disappearance is a rough measure as moisture content varies from food presented to that left over which affects weight.

**Necropsy and tissue sampling.** Immediately following euthanasia, the colon was removed, incised longitudinally, fecal material gently removed, and the colon was spiraled and frozen in a cassette with O.C.T (Optimal Cutting Temperature) compound (VWR, Radnor, PA). Tissues were stored at -80°C until frozen sectioning.
**Microdissection and RNA collection and processing.** Blocks of tissue were equilibrated to -20°C and tissues sectioned to 10 microns, using a cryostat microtome, with chuck and cabin maintained at -20°C. Tissue sections were collected onto SuperfrostPlus microscope slides and placed at -80°C until staining and laser microdissection was performed. Tissue sections were stained in small groups using the Arcturus HistoGene LCM frozen section staining kit (Life Technologies, Grand Island, NY) and stored in xylene until laser microdissection was performed. A PixCell II Laser Capture Microdissection System (Life Technologies, Grand Island, NY) was used to isolate epithelial cells of the deep region of the crypt, up to the +3 cells. Only microdissected cells of the colonic crypts were collected, to prevent an averaging of mRNA expression levels by other cell types as is the case in a whole tissue analysis. Cells were collected uniformly from all regions of the colon. RNA extraction was performed according to the PicoPure RNA Isolation protocol (Life Technologies, Grand Island, NY) using the DNA nuclease kit (QIAGEN, Valencia, CA). A Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) was used to confirm the quantity and quality of the isolated RNA.

**Semi-qualitative real-time PCR.** Reverse transcription was performed on the DNA-free RNA collected from the epithelial crypts of the colon using iScript cDNA synthesis kit (BioRad, Hercules, CA). Six genes (β-catenin, APC, Wnt 5a, Axin1, SFRP4, and TFRC) were selected for quantitative real-time PCR analysis (qRT-PCR) to investigate changes in the regulation of the Wnt signaling pathway among the different diets (Control, HA7, OS-HA7, and RS5) and treatments (Saline v AOM) (Table 3). GAPDH was used as a reference gene as our pilot studies demonstrated relatively uniform expression of this gene as compared with PSMB6 and PMM1.
(data not shown), which was in accordance with previous reports (Rubie, et al., 2005). Primer Quest software (Integrated DNA Technologies, Inc., Coralville, IA) was used to design the primers for qRT-PCR and were synthesized by Integrated DNA Technologies, Inc. SYBR Green Supermix kit (BioRad, Hercules, CA) was used for the qRT-PCR procedure according to the manufacturer's instructions. A GeneAmp 5700 Sequence Detection System was used with conditions of 95 °C for 3 min, and 45 cycles of 95 °C for 15 s, annealing temperature of the primer for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min, and 55 °C for 1 min.

**Statistical Analysis.** Body weight data for weeks 0 to 6 were analyzed using simple linear regression (weight to week) to get the slope coefficient for each mouse and then the student's t-test was applied to compare the slope coefficients for the saline and AOM groups. For weeks 6 to 16, the weight gain for each mouse was first calculated, and then ANOVA (overall F-test) was used to compare the average weight gain among the 8 groups. ΔCq values from PCR data from crypt epithelial populations were calculated (Bustin, et al., 2009; Lefever, et al., 2009). ΔΔCq values used to calculate fold-change as described by Pfaffl et al. (Table 5). Differences in ΔCq values were analyzed using Two-Way ANOVA with post-test (Two-way analysis of variance and Tukey’s multiple comparison test for normally distributed data or Kruskal-Wallis test and Dunn’s multiple comparison test for data that was not normally distributed) (Table 4) and Student's t-tests. All values were reported as mean ± standard deviation with the exception of fold change. A p-value < 0.1 was considered significant due to low sample volume (Casson, et al., 2005; Cai and Lashbrook, 2006). Data analysis was performed using SAS and/or GraphPad Prism.
RESULTS

Resistant Starch Content of the Experimental Diets. Resistant starch content of the Control, HA7, OS-HA7, and SA-HA7 diets based on the dry diet weight of the diets are listed in Table 2. The contents were calculated using both the AOAC 991.43 and Englyst Methods. The water content of each of the four diets was 12±0.2% (Table 2).

Body Weight. The body weights of the mice were statistically different when comparing the data from weeks 0-6, before diets were started, between the AOM- and saline-treated mice (p<0.0001) with the AOM-injected mice weighing less than the saline-injected mice. After the mice were placed on their respective diets, the total weight gains among diet and treatment groups were significantly different (p<0.001). The mice fed control diet were the heaviest, with those injected with AOM weighing significantly less than those injected with saline. The mice fed the OS-HA7 diet weighed the least, with mice fed this diet and injected with saline weighing more than those injected with AOM (Figure 1).

Expression of Wnt pathway genes by deep-crypt colonocytes. β-catenin mRNA expression was decreased among all AOM-treated mice compared with the saline counterparts within the CS, HA7, and OS-HA7 diets, with the decrease in the mice fed the OS-HA7 diet being significant (p=0.02). The AOM-treated mice fed the SA-HA7 diet had somewhat higher β-catenin mRNA expression than the saline-treated mice fed the SA-HA7 diet (p=0.14). The AOM-treated mice fed the OS-HA7 diet also had a significantly decreased mRNA expression of β-catenin when compared with the CS-fed mice injected with AOM (p=0.08) (Figure 2A). APC mRNA expression is relatively stable when comparing results between AOM-treated and saline-
treated mice within each diet. The AOM-treated mice fed the OS-HA7 diet also had a significantly increased mRNA expression of \( \text{APC} \) when compared with the CS-fed mice injected with AOM (\( p=0.003 \)) (Figure 2B). Expression of \( \text{Wnt 5a} \) mRNA was somewhat increased in the AOM-treated mice compared to the saline-treated mice within the OS-HA7 diet (\( p=0.14 \)). The AOM-treated mice fed the OS-HA7 diet had a significantly increased mRNA expression of \( \text{Wnt 5a} \) when compared to the CS-fed mice injected with AOM (\( p=0.004 \)). Expression was, however, somewhat decreased in the AOM-treated mice compared to the saline treated mice within the SA-HA7 diet (\( p=0.12 \)) (Figure 2C). \( \text{Axin 1} \) mRNA expression demonstrates varying mRNA levels with mice injected with AOM displaying an increased expression compared to saline-injected mice among the CS (\( p=0.28 \)) and SA-HA7 (\( p=0.09 \)) fed mice. The saline-treated mice fed the OS-HA7 diet had an almost significantly increased mRNA expression of \( \text{Axin 1} \) when compared with the CS-fed mice injected with saline (\( p=0.11 \)) (Figure 2D). \( \text{SFRP4} \) mRNA expression was increased in the AOM-treated mice fed both the OS-HA7 and SA-HA7 diets when compared with the CS-fed mice injected with AOM (\( p=0.03 \) and \( p=0.09 \), respectively) (Figure 2E). There were no significant differences in \( \Delta Cq \) results for mRNA encoding \( \text{TFRC} \) when comparing AOM-injected mice to saline-injected mice or when comparing data between diet groups (Figure 2F).

**DISCUSSION**

The inclusion of a highly resistant starch, SA-HA7, in the diet of mice in the preneoplastic stage of colon cancer resulted in a significant increase in \( \beta\text{-catenin} \) mRNA levels in the colonic epithelium. While a large body of evidence demonstrates that increased \( \beta\text{-catenin} \) mRNA levels are indicative of an active \( \text{Wnt} \) signaling pathway (Polakis, 1999; Polakis, 2000;
Hirose, et al., 2003; Feng Han, et al., 2006; Segditsas and Tomlinson, 2006), recent research may change the way in which we think about this pathway. Kwon et al., recently proposed an alternate model in which increased expression of $\beta$-catenin mRNA may instead induce post-translational modifications of $\beta$-catenin. These modifications are then hypothesized to inhibit proliferation of stem and progenitor cell populations through the Notch signaling pathway (Kwon, et al., 2011). This alternative model correlates to our mRNA expression data of other key genes, $Wnt\ 5a$ and $Axin1$, in the $Wnt$ signaling pathway, which also point toward a potentially inactive state of the $Wnt$ signaling pathway in SA-HA7-fed rats injected with AOM. However, our parallel studies showed the highest number of potentially pre-cancerous lesions in the SA-HA7 group, trending toward significance (Nelson, et al., unpublished).

The inclusion of a resistant starch with a relatively low level of resistance, OS-HA7, in the diet of AOM-initiated mice resulted in a significant decrease in $\beta$-catenin mRNA expression, which commonly is indicative of an inactive $Wnt$ pathway (Polakis, 1999). In contrast, it is possible that a secondary mechanism, acting through post-translational modifications of $\beta$-catenin by Notch signaling, is interacting with and regulating the $\beta$-catenin mRNA expression (Kwon, et al., 2011). This alternative model correlates to our mRNA expression data of other key genes, $APC$, $Wnt\ 5a$ and $SFRP4$, in the $Wnt$ signaling pathway, which suggest an active state of the $Wnt$ signaling pathway in OS-HA7-fed rats injected with AOM.

The resistant starch content of each diet was measured by both the AOAC 991.43 and Englyst Method as the AOAC 991.43 method does not measure the resistant starch content of the OS-HA7 diet as effectively as that of the Englyst Method, although no single method has been shown to be completely accurate (Ai, et al., 2013). The SA-HA7 diet was confirmed to have a statistically higher resistant starch content ($p<0.05$) than all other diets used in this study, which
may be causing enhanced fermentation in the mice fed this diet, probably due to increased amounts of starch available for the colonic microbiota to use as a food source.

The mice injected with AOM weighed significantly less than control mice injected with saline (p<0.05). At the time of injections, all mice were fed the highly digestible, commercially available cornstarch diet. Injection of AOM, a chemical carcinogen, is a stressful event for mice. A decrease in bodyweight of the mice demonstrates that they are reacting to the chemical carcinogen (Bissahoyo, et al., 2005; Suzuki, et al., 2006; Chen and Huang, 2009; De Robertis, et al., 2011). The mice did regain the lost weight and further increased weights after injections ceased demonstrating that the mice were able to thrive on these diets. After AOM or saline injections, mice were randomly divided into four diet groups, resulting in eight total groups of mice (a group of AOM-injected and a group of saline-injected mice per each diet group). The weight gain difference (weight at sacrifice minus the weight of the mice at start of the four diets) was statistically different among all eight groups (p<0.05) (Figure 1). The use of azoxymethane has also been shown to initiate the Wnt signaling pathway and cause an increase in cell proliferation (Hirose, et al., 2003; Takahashi and Wakabayashi, 2004; Chen and Huang, 2009). In our study, we observed no significant changes in gene expression, comparing AOM-injected with saline-injected, among the control diet-fed mice for any of the genes selected.

Diets containing moderate or high levels of resistant starch were able to modify mRNA expression of genes of the Wnt signaling pathway. OS-HA7 starch fed to AOM-injected mice increased APC, Wnt 5a, and SFRP4 mRNA levels relative to the CS-fed mice injected with AOM (p=0.003, p=0.004, and p=0.03, respectively). The mRNA expression levels of many of these genes were not only up-regulated when comparing with the control-fed mice, but also when comparing saline-injected mice with AOM-injected mice within the OS-HA7 diet.
Expression of *Wnt 5a*, SFRP4, and *TFRC* mRNA levels were somewhat increased in the AOM-injected mice fed the OS-HA7 diet relative to those injected with saline (p=0.14, p=0.17, and p=0.19, respectively). *β-catenin* mRNA expression was decreased in the AOM-injected mice fed the OS-HA7 diet relative to those injected with saline (p=0.02). The ability of the OS-HA7 diet to somewhat increase mRNA expression of *Wnt 5a*, SFRP4, and *TFRC* suggests that OS-HA7 fed in the diet may be able to up-regulate the *Wnt* signaling pathway in an initiated-state. While *β-catenin* mRNA expression suggests a down-regulation of the *Wnt* signaling pathway in AOM-injected mice, it is possible that another un-identified underlying mechanism is interacting with members of the *Wnt* pathway and regulating *β-catenin* mRNA expression. This study focused on the very earliest, preneoplastic stage of colon cancer, initiation, but the ability of a moderately resistant starch, OS-HA7, in the diet to increase mRNA expression levels show the potential to enhance cell proliferation (Fujise, et al., 2006; Kim and Milner, 2007).

A highly resistant starch, SA-HA7, created by conjugating stearic acid to a high amylose starch, in the diet has also demonstrated the ability to effectively decrease mRNA expression of genes in the *Wnt* pathway in crypt epithelial cells of the colon. SA-HA7 fed in the diet has demonstrated an ability to somewhat reduce *Wnt5a* mRNA expression in AOM-injected mice relative to those injected with saline (p=0.12). Mice injected with AOM also have an increased *Axin1* mRNA expression compared with those injected with saline among the SA-HA7 diet group (p=0.09). Both of the mRNA expression levels demonstrate some ability of the SA-HA7 diet to down-regulate the *Wnt* signaling pathway. However, SA-HA7 fed in the diet of mice injected with AOM had a somewhat increased *β-catenin* mRNA expression relative to the saline-injected mice fed the SA-HA7 diet (p=0.14). Historically, increased *β-catenin* levels have been shown to be indicative of an active *Wnt* signaling pathway, but this data does not correspond to
the mRNA expression of other genes tested. However, this data does correlate to parallel studies demonstrating the greatest ACF in the SA-HA7 fed mice injected with AOM (Nelson, et al., unpublished). This increase in $\beta$-catenin mRNA levels does correspond to the expression data of other genes if a recently proposed alternate model, in which increased expression of $\beta$-catenin mRNA may instead induce post-translational modifications of $\beta$-catenin, is used. The modification of $\beta$-catenin would then inhibit proliferation of stem and progenitor cells through the Notch signaling pathway (Kwon, et al., 2011). This alternative model more readily correlates to our mRNA expression data of other key genes in the Wnt signaling pathway, which also point toward a potentially inactive state of the Wnt signaling pathway in SA-HA7-fed mice injected with AOM. This result has also been supported by other research suggesting certain types of dietary resistant starch inhibit Wnt signaling activity (Wollowski, et al., 2001; Fujise, et al., 2006; Kim and Milner, 2007; Nofrarias, et al., 2007; Fung, et al., 2012). Though this alternate model does not correlate to ACF results by Nelson et. al., the validity of ACF as a preneoplastic lesion has recently come in to question (Hirose, et al., 2003).

Overall, these results demonstrate that the OS-HA7 diet, containing a low level of resistant content, was able to up- and down-regulate the expression of the mRNA for $\beta$-catenin, APC, Wnt 5a, SFRP4, and TFRC of the Wnt signaling pathway in the stem cell niche. This suggests that a low level of resistant starch content may not slow cell proliferation and differentiation and may instead increase cell proliferation, which may potentiate the development of colorectal cancer.

On the other hand, the results also demonstrate that the SA-HA7 diet, containing a high amount of resistant starch, has the potential to effectively modify the expression of mRNA encoding genes for $\beta$-catenin, Wnt 5a, and Axin 1 in the colonic crypt cells. With the exception
β-catenin mRNA expression, all data suggest an ability of SA-HA7 fed in the diet to inhibit the Wnt signaling pathway. An alternate model has been recently proposed in which increased expression of β-catenin mRNA induces post-translational modifications of β-catenin is able to inhibit proliferation of stem and progenitor cells through the Notch signaling pathway. If this model is considered, then the increase β-catenin mRNA expression seen in this study may also be indicative of a down-regulated Wnt signaling pathway in an AOM-induced state. This suggests an ability of resistant starch to decrease the mRNA expression of key genes of the Wnt signaling pathway.

This study demonstrates the importance of the percentage of resistant content in the diet, as these four diets, with significantly different resistant starch contents, gave very different ΔCq results. Our studies suggest that enhanced intake of high content resistant starch has the potential to help to inhibit the Wnt signaling pathway and provide many health benefits, while a low content of resistant starch may actually have harmful effects. The genes in these studies may also be prospective targets for the prevention of colorectal cancer, with future studies needed to investigate the effects of both AOM and diet on the protein levels of the Wnt signaling pathway.

**ABBREVIATIONS**

AOM, Azoxymethane
RS, Resistant Starch
HA7, High-Amylose 7 starch diet
OS-HA7, Octenyl succinate acid starch diet
SA-HA7, Stearic acid complexed starch diet
CRC, Colorectal cancer
LCM, Laser Capture Microdissection

O.C.T, Optimal Cutting Temperature

SAFETY

Azoxymethane is a potent carcinogen used experimentally to induce colon cancer in rats and mice. It might cause harm to human if swallowed, inhaled, or absorbed through skin.

ACKNOWLEDGEMENTS

This research was funded by Plant Sciences Institute at Iowa State University and the United States Department of Agriculture (USDA) NRI/AFRI Project “Effects of lipids on physical properties, digestibility and nutritional benefits of starchy foods.” We appreciate the contributions of Toni Christofferson for histopathology processing and sectioning, Margie Carter for assistance with LCM, and to Dr. Angela Pillatzki for assisting in optimizing research protocols. We include a very special thank you to Reed Faldet, Catie Mullen, Dana Pralle, and Kylie Thompson for the extensive help with the care of the mice.

REFERENCES


FIGURE LEGENDS

Figure 1. Graph of bodyweights of each treatment group. Each AOM group contained 10 mice, whereas each saline group contained 5 mice, with the exception of CS-Saline (n=4). *represents statistical significant differences of the OS-HA7 group injected with AOM compared with both the OS-HA7 group injected with saline and the CS group injected with AOM from the start of the experimental diets (week 7) until sacrifice (week 17), with only weeks 8, 12, and 17 marked as a representation.

Figure 2A-F. mRNA expression of Wnt pathway-related genes in the colonic crypt of mice fed diets containing CS, HA7, OS-HA7, or SA-HA7 starches. Data reported are ∆Cq values, using the ∆∆Cq method. Each AOM group had n=10 while each saline group had n=5 with the exception of CS-saline which had n=4. Bars represent statistical significance with one star (*) representing p-value <0.1 and two stars (**) represent p<0.05. + bars represent a trend toward statistical significance (p<0.12). P-values of the Two-Way ANOVA analysis for each mRNA species are listed in Table 5.
FIGURES

Figure 1.

Average body weights of treatment groups per week
Figure 2

A. \(\beta\)-catenin mRNA

B. APC mRNA
Figure 2 Continued

**C**

Wnt 5a mRNA

**D**

Axin 1 mRNA
Figure 2 Continued

E  SFRP4 mRNA

F  TFRC mRNA
TABLE LEGENDS

Table 1. Diet Ingredients. Components of the starch diet as characterized by Zhao, et al. (in press 2014).

Table 2. Resistant Starch and Water Content of Starches and Diets. Percentage resistant starch content of the diet was determined using both the AOAC method 991.43 and the Englyst Method.

Table 3. Primer Sequences for Quantitative RT-PCR of Mouse Colon Samples.

Table 4. Modulation of mRNA expression of genes of the Wnt pathway by dietary resistant starch in the colonic crypts of AOM-injected and control mice. With comparisons made within and across diets ΔΔCq method. *indicates statistical significance (p<0.1).

Table 5. P-values of Two-Way ANOVA of genes of the Wnt pathway in colonic crypts.
TABLES

Table 1: Diet Ingredients

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
<th>Diet Components</th>
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<tbody>
<tr>
<td>starch</td>
<td>55.0%</td>
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<tr>
<td>casein</td>
<td>20.0%</td>
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<tr>
<td>dextrose</td>
<td>15.0%</td>
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<tr>
<td>cellulose (insoluble fiber)</td>
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<tr>
<td>mineral mix (AIN-93)</td>
<td>3.5%</td>
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<td>choline</td>
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</tr>
<tr>
<td>methionine</td>
<td>0.3%</td>
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<tr>
<td>vitamin mix (AIN-93)</td>
<td>1.0%</td>
</tr>
<tr>
<td>corn oil</td>
<td>5.0%</td>
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1: Diets were prepared as described in the text. All non-starch diet ingredients were purchased from Harland Teklad (Madison, WI) or Spectrum (Gardena, CA).
Table 2: Resistant starch and water contents of the diets.

<table>
<thead>
<tr>
<th>Starch Groups</th>
<th>Resistant Starch Content of the Diets on a Dry Diet Basis Measured by AOAC method (n=2) (% ± sd)</th>
<th>Resistant Starch Content of the Diets on a Dry Diet Basis Measured by the Englyst Method (n=2) (% ± sd)</th>
<th>Water Content of the Diets (n=2) (% ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7±0.7%</td>
<td>1.40 ± 0.39%</td>
<td>12± 0.2%</td>
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<tr>
<td>HA7</td>
<td>19.0±1.8%</td>
<td>12.75 ± 0.20%</td>
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<td>OS-HA7</td>
<td>4.7±0.4%</td>
<td>15.28 ± 2.70%</td>
<td>12± 0.2%</td>
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<tr>
<td>SA-HA7</td>
<td>31.1±1.7%</td>
<td>17.63 ± 0.59%</td>
<td>12± 0.2%</td>
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Data are expressed as mean ± SD.
<table>
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<tr>
<th>Gene Name</th>
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<td>GAPDH</td>
<td>Forward: TCAACAGCAACTCCCACTCTTTCCA</td>
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<tr>
<td></td>
<td>Reverse: ACCCTGTTGCTGTAGCGTATTCA</td>
</tr>
<tr>
<td>APC</td>
<td>Forward: TGCACAACCTTTGTGCCCTTTGGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTCTGATGGCTCTCAACCAGGACA</td>
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<tr>
<td>Axin1</td>
<td>Forward: AGAAACACATGGTCATGCCAAGCC</td>
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<td></td>
<td>Reverse: TCCCAGATTCAGCCTTTTGGGT</td>
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<td>Beta-Catenin</td>
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<td></td>
<td>Reverse: AGATGCCAGGCTCATGATGTTT</td>
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<td>SFRP 4</td>
<td>Forward: TCAATCCAATTTCTTCTGCTG</td>
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<td>Reverse: AGATCCAGGCTTCTGCTGCTGCTGT</td>
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<td>TFRC</td>
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<td></td>
<td>Reverse: GCTGCTGATGCTGTGAAGCTT</td>
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<tr>
<td>Wnt 5a</td>
<td>Forward: ACTGGCAGGACTTTCTCAGGACA</td>
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<tr>
<td></td>
<td>Reverse: GCCTATTTGCATCCCTGCCAAA</td>
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Table 4. Modulation of mRNA expression of genes of the Wnt pathway by dietary resistant starch in the colonic crypt of AOM-injected and control mice.

<table>
<thead>
<tr>
<th>Effect of Dietary RS on gene expression in saline treated colon (b)</th>
<th>Diets (a)</th>
<th>β-catenin</th>
<th>APC</th>
<th>Wnt 5a</th>
<th>Axin 1</th>
<th>SFRP4</th>
<th>TFRC</th>
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<td>HA7</td>
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<td>0.02</td>
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a= animals not treated with AOM; ΔΔCqs calculated comparing test diets to control (CS) diet
b= fold change

<table>
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<tr>
<th>Effect of Dietary RS on gene expression in AOM-induced colon (b)</th>
<th>Diets (c)</th>
<th>β-catenin</th>
<th>APC</th>
<th>Wnt 5a</th>
<th>Axin 1</th>
<th>SFRP4</th>
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<tr>
<td>HA7</td>
<td>1.96</td>
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<td>OS-HA7</td>
<td>5.64*</td>
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<td>0.37</td>
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b= fold change
c= animals treated with AOM; ΔΔCqs calculated comparing test diets to control (CS) diet

<table>
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<tr>
<th>Effect of AOM-induction on gene expression in the colon (b)</th>
<th>Treatments (d)</th>
<th>β-catenin</th>
<th>APC</th>
<th>Wnt 5a</th>
<th>Axin 1</th>
<th>SFRP4</th>
<th>TFRC</th>
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<tr>
<td>CS</td>
<td>1.20</td>
<td>0.35</td>
<td>0.30</td>
<td>0.23</td>
<td>2.22</td>
<td>1.89</td>
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<tr>
<td>HA7</td>
<td>3.36</td>
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<td>OS-HA7</td>
<td>3.89*</td>
<td>0.38</td>
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<td>SA-HA7</td>
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<td>1.13</td>
<td>12.61</td>
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<td>1.57</td>
<td>3.83</td>
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b= fold change
d= ΔΔCqs calculated comparing AOM-treatment with saline-treatment for each diet
Table 5. P-values of Two-Way ANOVA of genes of the Wnt pathway in colonic crypts

<table>
<thead>
<tr>
<th>Interaction</th>
<th>β-catenin</th>
<th>APC</th>
<th>Wnt 5a</th>
<th>Axin 1</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Diet (a)</td>
<td>0.22</td>
<td>0.90</td>
<td>0.20</td>
<td>0.27</td>
<td>0.59</td>
<td>0.83</td>
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<tr>
<td>Treatment (b)</td>
<td>0.65</td>
<td>0.01</td>
<td>0.06</td>
<td>0.78</td>
<td>0.09</td>
<td>0.67</td>
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<tr>
<td>Treatment (b)</td>
<td>0.87</td>
<td>0.60</td>
<td>0.94</td>
<td>0.58</td>
<td>0.43</td>
<td>0.61</td>
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</tbody>
</table>

a=p-values of the effect of the diet with p<0.1 being significant
b=p-values of the effect of either saline or AOM injection with p<0.1 being significant
CHAPTER 4: GENERAL CONCLUSIONS

General Discussion

With approximately 102,480 new cases of colon cancer in 2013, colorectal cancer (CRC) is the third most commonly diagnosed cancer in the USA alone (Society, 2013). While the number of people dying from colorectal cancer has slowly begun to decline, due to an increased screening rate, the use of dietary preventative measures holds the possibility of drastically reducing the incidence of this disease (Clausen, et al., 1991; Lattimer and Haub, 2010). Dietary resistant starch (RS) shows promise as a prebiotic agent for preventing colorectal cancer by modifying proliferation and maturation in colonic crypts, the site of intestinal mucosal renewal.

Resistant starch fermentation by colonic bacteria has been shown to be beneficial to human health (Cummings, et al., 1996). Butyrate, a fermentation product, is a short-chain fatty acid known to have protective effects on the epithelium of the colon. It has been shown to be able to inhibit DNA synthesis of neoplastic cells through inhibition of histone deacetylases (Nofrarias, et al., 2007; Fung, et al., 2012). Butyrate and other products of colonic RS fermentation, in a molecular mechanism not fully understood, may regulate the Wnt signaling pathway. This pathway plays a major role in mammalian development and maintains many tissues and organs by regulating cell proliferation, differentiation, and epithelial-mesenchymal interactions (Moon, et al., 1997; Smalley and Dale, 1999). The Wnt pathway regulates and acts specifically on the stem cell populations, in each tissue, which lends to its role in initiation and progression of cancer, with colorectal and breast cancers being most influenced by this pathway (Polakis, 2000; Reya and Clevers, 2005).

In our studies, various diets containing resistant starches were tested for their modulatory effects on the Wnt signaling pathway, using mRNA expression of key genes of this pathway as
markers of overall pathway activity. The first study investigated the effect of diets containing an inbred, high-resistance corn line, GUAT, compared to a control inbred line, AR, with highly digestible starch, or a naturally hybridized line, ARxGUAT. Diets as fed containing these three starches had varying resistant contents with both the AR and ARxGUAT diets having a low level of digestion resistance, 5.4±0.5% and 8.6±0.6%, respectively, while the GUAT diet contained 1.6±0.9% resistant starch. This study, conducted in male F344 rats fed one of three test diets for 8 weeks following AOM-injection, investigated effects of a minor increase in resistant content (ARxGUAT) compared to control (AR) and a major increase in resistant content (GUAT) compared the control (AR) on mRNA expression of genes of the \textit{Wnt} pathway. Whole colon tissue samples were examined using qRT-PCR analysis for a panel of genes of the \textit{Wnt} pathway or their downstream targets. Epithelial cell populations of the colonic crypts and lining the luminal surface were also microdissected and the expression of two genes, \(\beta\)-\textit{catenin}, a transcription activator, and \textit{TFRC}, a target gene of the \textit{Wnt} pathway, was analyzed. This study also measured the incidence and multiplicity of a putative preneoplastic lesion, Aberrant Crypt Foci (ACF).

Results of this study demonstrated no statistical differences in number of putative mucosal lesions, aberrant crypt foci (ACF), among the three diets. It is worthwhile to note that the ability of this lesion to be a reliable marker of colorectal cancer has recently been questioned. In this study performed during the pre-neoplastic stage of colon cancer, we identified ACF only in AOM-treated rats, suggesting that ACF was induced as a primary or secondary result of AOM treatment, but ACFs have not been conclusively shown to represent a preneoplastic condition. There was, however, an increase in the average number of crypts per focus in AOM-induced rats fed a diet high in resistant starch (GUAT diet), compared with rats fed the readily digestible AR
diet. The absence of a significant difference in crypt multiplicity in colons of rats fed diets containing the highly digestible (AR) or moderately digestible (ARxGUAT) starch suggests a possible effect of resistant starch on crypt multiplicity (Wargovich, et al., 2010). Evaluation of gene expression using full-thickness samples of colon revealed some statistically significant differences in mRNA expression among the various diets, which may prove to be biologically significant data. Rats fed the control diet demonstrate an increased mRNA of Wnt 8a, HNF1α, and Axin2 expression in the AOM-initiated mice relative to the saline-injected mice. However, TFRC, SFRP4, and WISP-1 mRNA expression in the full-thickness samples are decreased in AOM-initiated mice relative to the saline-injected mice fed the control AR diet. Diets containing moderate or high levels of resistant starch also modify mRNA expression of genes of the Wnt signaling pathway. ARxGUAT starch fed to saline-injected mice decreased TFRC, SFRP4, and WISP-1 mRNA levels relative to the control AR fed mice injected with saline. Although these three genes play roles in multiple pathways (Rohrs, et al., 2009; Davies, et al., 2010), the ability of the ARxGUAT diet to decrease mRNA expression is still important. ARxGUAT fed in the diet not only reduces mRNA expression in saline-injected mice, but also in those injected with AOM. β-catenin, HNF1α, SFRP4, and WISP1 mRNA levels are all decreased in the full-thickness colonic tissue in AOM-injected rats fed the ARxGUAT diet relative to the control, AR, fed mice injected with AOM, suggesting that ARxGUAT fed in the diet may be able to down-regulate the Wnt signaling pathway in an initiated-state. This study only looked at the preneoplastic stage of colon cancer, but the ability of a moderately resistant starch, ARxGUAT, in the diet to decrease mRNA expression levels at this stage show the potential for inhibiting cell proliferation.
A highly resistant starch, GUAT, in the diet has also demonstrated ability to effectively decrease mRNA expression in the full-thickness colon samples and site specific, surface or crypt, epithelial cells of the colon. Mice fed the GUAT diet, injected with saline have decreased TFRC, Axin2, SFRP4, and WISP-1 full-thickness mRNA levels relative to the control, AR, fed mice injected with saline. These mRNA expression levels demonstrate that both low and high levels of resistant starch content in the diet are able to down-regulate the Wnt signaling pathway. GUAT in the diet is able to decrease mRNA expression of Axin2 in the full-thickness colon, which was not down-regulated by the ARxGUAT diet. The decrease of another gene is hypothesized to be due to the increased resistant starch content of GUAT and suggests that a higher level of resistant starch has a greater impact on maintaining the colon in a normal, non-initiated, state. GUAT fed in the diet does not only reduce mRNA expression of the full-thickness colon in saline-injected mice, but also in those injected with AOM. The Axin2 mRNA level of the full-thickness colon is decreased in AOM-injected rats fed the GUAT diet relative to the control, AR, fed rats injected with AOM. The decrease in Axin2 mRNA expression suggests an ability of this dietary resistant starch, GUAT, to not only influence expression in a saline-injected model, but also in an AOM-injected model. GUAT fed in the diet of rats injected with AOM also had a decreased β-catenin mRNA expression both the colonic epithelial surface and epithelial crypt relative to AOM-injected rats fed the AR, control, diet. β-catenin mRNA levels are also decreased in the AOM-injected rats fed the GUAT diet relative to the saline-injected rats fed the GUAT diet. Decreased β-catenin levels have been shown to be indicative of a non-active Wnt signaling pathway. This decrease in β-catenin mRNA levels of the full-thickness tissue and of the niche specific cells correlates to our mRNA expression data of other key genes in the Wnt
signaling pathway, which also point toward a potentially inactive state of the Wnt signaling pathway in GUAT-fed rats injected with AOM.

Overall, these results demonstrate that increased levels of dietary resistant starch were able to modify the expression of genes encoding β-catenin, TFRC, Axin2, SFRP4, and WISP-1 of the Wnt signaling pathway in both the full-thickness colon and in the stem cell niche and mature surface colonocytes of rats injected with AOM and fed the ARxGUAT or GUAT diet. The GUAT diet was able to decrease the mRNA expression of a few more genes than that of the ARxGUAT diet in AOM-injected rats suggesting that the level of resistant content is important and that a higher resistant content may be able to decrease overall Wnt signaling to a greater degree.

With the exception the increased ACF multiplicity, mRNA data suggest an ability of GUAT fed in the diet to inhibit the Wnt signaling pathway. This suggests an ability of resistant starch to decrease the mRNA expression of key genes of the Wnt signaling pathway (Giles, et al., 2003; Segditsas and Tomlinson, 2006).

We also demonstrated the important differences in gene expression patterns between samples derived from whole tissue and discrete populations of cells residing in different mucosal microenvironments. This highlights the importance of evaluating mRNA levels from specific populations of cells.

The second study looked at effects of three diets rich in resistant starch of three different classifications compared to a control, highly digestible corn starch diet. Diets containing HA7 (RS type 2), OS-HA7 (RS type 4), SA-HA7 (RS type 5) or a control corn starch (CS) were fed to male A/J mice for 10 weeks following 4 weekly IP injections of either azoxymethane (AOM) or physiological saline. Levels of mRNA expression of several genes of the Wnt pathway or
downstream targets were analyzed from populations of cells deep within the colonic crypts to evaluate the individual efficacy of the different resistant starches to modulate this pathway. The inclusion of a starch with minimal resistance, OS-HA7, in the diet of mice in the preneoplastic stage of colon cancer resulted in a significant increase in the mRNA expression APC, Wnt 5a and SFRP4 relative to the mice fed control diet, which may indicate an increase in the Wnt signaling pathway activity. Although β-catenin mRNA expression did not follow this increase, it is possible that a secondary mechanism is interacting with and regulating the β-catenin mRNA expression (Kwon, et al., 2011). The inclusion of a highly resistant starch, SA-HA7, in the diet at the same stage of carcinogenesis resulted in almost significant decreases in mRNA expression of Wnt 5a and a significant increase in mRNA encoding an antagonist, Axin 1, in the AOM-injected mice relative to the saline-injected mice. β-catenin mRNA expression of the SA-HA7-fed mice also did not follow the trend and display a decrease in expression, but instead had an increase. This increase in expression does correspond to both the Wnt 5a and Axin 1 expression based on recently reported research, suggesting that excessive translocation of β-catenin to the nucleus may induce reduced proliferation of the stem and progenitor cell populations and induction of Notch signaling as has been recently suggested (Hirata, et al., 2013). This mechanism is thought to control proliferation by post-translational inactivation of β-catenin and would not be detected at the mRNA expression level (Kwon, et al., 2011).

Overall, these results demonstrate that the OS-HA7 diet, containing a low level of resistant content, was able to both up- and down-regulate the expression of the mRNA for β-catenin, APC, Wnt 5a, SFRP4, and TFRC of the Wnt signaling pathway in the stem cell niche. This suggests that a low level of resistant starch content may not slow cell proliferation and differentiation and may instead increase cell proliferation, which may therefore potentiate the
development of colorectal cancer. On the other hand, the results also demonstrate that the SA-HA7 diet, containing a high amount of resistant starch, has the potential to effectively modify the expression of mRNA encoding genes for $\beta$-catenin, Wnt 5a, and Axin 1 in the colonic crypt cells. With the exception $\beta$-catenin mRNA expression, all data suggest an ability of SA-HA7 fed in the diet to inhibit the Wnt signaling pathway. An alternate model of regulation of $\beta$-catenin activity has been proposed recently in which increased expression of $\beta$-catenin mRNA induces post-translational modifications of $\beta$-catenin able to inhibit proliferation of stem and progenitor cells through the Notch signaling pathway. If this model is considered, then the increase $\beta$-catenin mRNA expression seen in this study may also be indicative of a down-regulated Wnt signaling pathway in an AOM-induced state. This suggests an ability of resistant starch to decrease the mRNA expression of key genes of the Wnt signaling pathway.

These studies used rodent models of human disease, with model selection being crucial for accurate reflection of the disease in human beings. When studying the effects of diet on sporadic colorectal cancer, mouse models with inbred genetic defects are not appropriate for investigation of sporadic CRC, as they better mimic inherited forms of human CRC. Chemically induced CRC models, like AOM-treatment of A/J mice, are better suited for these studies, as they better correlate to the sporadic colorectal cancer model mimicking a spontaneous DNA damage and initiation of carcinogenesis (Hung, et al., 2010). A/J mice show a dose-dependent response to azoxymethane (AOM) and the combination of A/J mice injected with AOM as the carcinogen is optimal for this study and further strengthened by the fact that AOM is specific for inducing carcinogenesis in the colonic tissue (Bissahoyo, et al., 2005).

Both experiments in this project investigated the effects of dietary resistant starch on the Wnt signaling pathway, a regulator of colonic carcinogenesis. To do this, several key genes of
the Wnt pathway were chosen for quantative real-time PCR (qRT-PCR). APC is a tumor suppressor gene that acts as a downstream regulator of the Wnt pathway by controlling cellular levels of β-catenin (Dimitriadis, et al., 2001). APC is able to bind both Axin and β-catenin which allows for phosphyloration and subsequently degradation of β-catenin. This prevents translocation of β-catenin to the nucleus and initiation of transcription of genes required for cellular proliferation (Shibata, 1997). Defects in regions of either APC or β-catenin that are critical to binding will result in a lack of inhibitory binding of β-catenin and subsequent uncontrolled transcription. Transferrin Receptor Protein (TFRC) is a target of the Wnt pathway is required for delivery of iron from transferrin to cells, a function that is necessary for cell survival. In an unregulated growth state, such as cancer, TFRC expression would be greatly enhanced (Ryschich, et al., 2004). Most genes of the Wnt pathway that we measured were up-regulated by AOM treatment and most were down-regulated by diets containing the highly resistant starches, GUAT and SA-HA7. Axin, however, is an antagonist of the Wnt pathway whose function is to organize a complex between itself, APC, and β-catenin promoting the phosphyloration and degradation of β-catenin. An up-regulation of this protein results in a decreased expression of β-catenin, inhibition of the Wnt pathway and thus decreased cellular proliferation rates (Polakis, 2000; Leung, et al., 2002). This is consistent with both our studies, with reduced Axin inhibition of Wnt in rats fed diets containing the highest levels of resistant starch, GUAT and SA-HA7.

In conclusion, we demonstrate that a high level of resistant starch content in the diet modulates the expression of mRNA species integral to the Wnt signaling pathway expression in two AOM-induced animal models. Our studies suggest that enhanced intake of resistant starch has the potential to inhibit the Wnt signaling pathway and to provide many health benefits. These
studies provide the first research documenting population- and context-specific modulatory
effects of resistant starches on genes of the Wnt signaling pathway. The genes in these studies
may also be prospective targets for the prevention of colorectal cancer, with future studies
needed to investigate the effects of both AOM and diet on the Wnt signaling pathway at the
protein and functional levels.

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APPENDIX A. EXPERIMENTAL PROTOCOLS

Title: Starch cooking by water boiling method and diet preparation
Lab group: Birt/Whitley lab
Updated by: Bridget Nelson and Nicole Cray 5-31-13

The following SOP outlines the methods for cooking starches and diet preparations.

Starch and Ingredient Storage:
Starches are stored in closed tubs and stored at 40°F in the HNSB 2007 walk in cooler. Diet ingredients and sealed and stored at 40°F in the HNSB LAR diet ingredients coolers. Choline and corn oil are stored at room temperature.

Starch Cooking:
Cooking processes conducted in 2007 HNSB. Starch needed is weighed in a stainless steel pot. Three parts water are added for every part of starch weighed out (1 part starch: 3 parts water). The OS-HA7 starch uses 3 parts water minus 100ml (for dry starch amounts up to 2000g). A wooden spoon is used to constantly stir the starch over medium to high heat until starch is cooked to the final endpoints, noted below (Figures 1-4). Note that a plastic spoon will melt. Once the final cooked form is observed, the starch is placed onto labeled pieces of aluminum foil. Cooking times vary between starch types and depend on the amount of starch being cooked. Generally, CS cooks fastest, followed by OS-HA7, SA-HA7, and then finally HA7. HA7 burns quickly and needs to be cooked at a lower temperature setting (medium low to medium). The cooked starches are then transported to the HNSB LAR diet preparation room.

Figure 1: Cooked Control starch
Figure 2: Cooked HA7 starch
Diet Preparation:
Diet ingredient amounts are calculated based on the percentages presented in Table 1. If the amount of starch made is less than 500g, mixing by hand is preferred over the standard mixer. All dry ingredients (not starch) are measured and poured into a plastic mixing bowl. Corn oil is measured last and added last. All ingredients are mixed until homogeneous and the final product is dough-like. Trays are covered with tin foil and labeled with the diet name, lab name, and date prepared for each diet. The diet dough mixture is placed onto the foil covered trays and pressed to various thicknesses: CS-1 inch, HA7-1-1.5”, OS-HA7-2-2.5”, SA-HA7-1-1.5”. The diets are left on metal shelves overnight with a fan on a medium setting directed at the shelves (Figures 5-8).

Table 1: Diet ingredient components

<table>
<thead>
<tr>
<th>Diet Component</th>
<th>Percentage of Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>55%</td>
</tr>
<tr>
<td>Casein</td>
<td>20%</td>
</tr>
<tr>
<td>Dextrose</td>
<td>15%</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>3.5%</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1%</td>
</tr>
<tr>
<td>Choline</td>
<td>0.2%</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.3%</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>5%</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 6: HA7 diet after overnight drying a) top view b) side view, compressed to 1.5 inches

Figure 7: OS-HA7 diet after overnight driving a) top view b) side view, compressed to 2 inches

Figure 8: SA-HA7 diet after overnight drying a) top view b) side view, compressed to 1.5 inches
The following SOP outlines the methods for feeding animals the resistant starch diets.

1. Weigh and record total weight of each diet before feeding.
2. Based on the total weight, determine the amount that can be fed to each diet group of animals. The average amount of diet per animal per day should be approximately 10-15g, possibly more depending on the batch of mice and individuals within the group.
3. Measure portions of food for each animal based on the amount determined in Step 2 (Figure 1).
4. Weigh and record leftover diet not used to feed animals.
5. Refer to Figures 2-4 for adding diet portions to individual cages.
6. Check back the following day to check the food levels and add more food to animals that are low on food.

Figure 1: Measured out food portions for animal feeding.

Figure 2: Diet placed in cages before compression through metal grates.
Figure 3: Compressed diet

Figure 4: Alternate view of compressed diet
The following SOP outlines the method for staining frozen slides for LCM following the Arcturus HistoGene LCM Frozen Section Staining Kit (Life Technologies, Grand Island, NY) Protocol.

Slides stored at -80°C until ready

Allow slides to warm up which will take approximately 5 minutes

Then immerse into slide tubes
75% Ethanol 30seconds
DH2O 30seconds
HistoGene Stain 20seconds
DH2O 30seconds
75% Ethanol 30seconds
95% Ethanol 10 dips
95% Ethanol 30seconds
100% Ethanol 10 dips
100% Ethanol 30seconds
Xylene 2 1/2 minutes

Then transfer the slides to a fresh slide tube of xylene for transport to the LCM machine in 119 Molecular Biology.

NOTE: This procedure needs to be done in the upmost care to avoid RNA degradation. Only do experiments in an area dedicated to RNase free procedures. Always wear gloves and use RNase Zap (Life Technologies, Grand Island, NY) liberally.
The following SOP outlines the method for collecting specific populations of cells using the Arcturus PixCell II Laser Capture Microdissection System (LCM) (Life Technologies, Grand Island, NY) in 119 Molecular Biology.

What is needed:
Arcturus CapSure HS LCM Caps (Life Technologies, Grand Island, NY)
Extraction buffer from the Arcturus PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY)
RNase spray
Kim Wipes
Pipette (10ul)
Pipette tips
Desiccator
Forceps
Gloves
Heat Block
Cap Tray that fits the heat block
0.5 mL tubes that fit over the cap system
SAMPLES (carefully transfer in tubes of xylene)

Immediately remove slides from xylene and lay out in hood to dry (Room 114)
-time required depends on weather/humidity levels
-allow slides to dry completely

While slides are drying, wipe everything with RNase
-Be thorough
-As wiping, turn computer, microscope, power box, and tower on

Set Caps into Machine making sure they are secure

Set out all other supplies needed making sure to keep everything RNased

On computer, select the Ver 2.0.0 program
-Need to select a name and then an experiment

Get slide and place on scope stage
-focus the slide either through the oculars or on screen
-move cap onto slide
-turn on the laser and focus the laser so it is a defined dot

Test the strength of the laser by firing into an empty space on the slide
-Adjust the power and duration as needed. (Will need stronger/longer with wet slides.)

Collect as many cells of the population as possible
-want enough to fill the cap and do not be afraid to pick up the cap and move it.

When cap is full, lift off the slide and place into cap holder.
-put cap topper on and fill with 10μL of Extraction Buffer.
-put 0.5 mL tub on top and ensure whole system is secure.

Cover with heat block until all slides are collected.
-transport back to 2713 VetMed

Incubate at 42°C for 30 minutes
-taking care to watch for evaporation due to a loose fit.

Flip apparatus over and spin down at 800g for 1 minute.

Freeze immediately at -80°C OR continue to the RNA collection protocol
The following SOP outlines the methods for RNA isolation following cells collection via LCM.

**PicoPure RNA Isolation Kit from Arcturus (Life Technologies, Grand Island, NY) using the optional RNase-Free DNase Set from Qiagen (QIAGEN, Valencia, CA)**

1. Pipette 250uL conditioning buffer onto purification column filter membrane and incubate at Room Temperature for 5 minutes.
   a. Centrifuge at 16,000xg for 1 minute
2. Pipette 10uL 70% Ethanol into cell extract tube from LCM cell collection protocol.
   a. Mix by pipetting up and down and then transfer onto conditioned column (about 20uL).
3. Centrifuge column at 100xg for 2 minutes then 16,000xg for 30 seconds.
4. Pipette 100uL Wash Buffer 1 onto membrane and centrifuge at 8,000xg for 1 minute.

**OPTIONAL DNase Treatment**

I. Pipette 5uL DNase Stock Solution to 35uL Buffer RDD for each sample
   a. Mix by gentle inversion.
II. Pipette this 40uL mixture onto membrane and incubate at room temperature for 15 minutes.
III. Pipette 40uL Wash Buffer 1 (above kit) onto membrane and centrifuge at 8,000xg for 15 seconds.

5. Pipette 100uL Wash Buffer 2 onto membrane and centrifuge at 8,000xg for 1 minute.
6. Pipette another 100uL of Wash Buffer 2 onto membrane and centrifuge at 16,000xg for 3 minutes.
7. Transfer the column to a new 0.5mL tube (in kit).
8. Pipette 30uL Elution Buffer directly onto membrane (touch tip to membrane with care).
   a. Incubate at room temperature for 1 minute.
9. Carefully centrifuge at 1,000xg for 1 minute and then 16,000xg for 1 minute.
10. Remove membrane and close tube.
    a. Nanodrop then freeze.

Check RNA content using nanodrop (2758 VetMed) \(\sim\)25ng/ul (Note that this is low)
RNA samples store in -80°C freezer after the isolation
The following SOP outlines the methods for cDNA synthesis following RNA extraction.

**Up to 24 extracted/purified RNA samples can be converted to DNA in one run. Thaw RNA samples 3-5 minutes.**

**iSCRIPT cDNA synthesis kit from Bio-rad (BioRad, Hercules, CA)**

Add together:
4 ul 5x iScript Reaction Mix  
1 ul iScript Reverse Transcriptase  
15 minus X ul Nuclease free water  
X ul RNA template (for x amount of template: up to 1ug)  
Total=20 ul

Mix together in 0.2 ml thin wall tube and put in PCR thermal cycler machine

**Run at:**
5 min at 25 C  
30 min at 42 C  
5 min at 85 C  
Hold at 4 C

Use VetMed GeneAmp PCR System 2400 located in room 2605

**Protocol = USER: Nicole METHOD: cDNA**

Check DNA content using nanodrop (2758 VetMed) \( \rightarrow \sim 1200-1500\text{ng/ul} \)  
cDNA samples store in -80°C freezer after the synthesis
Title: qRT-PCR
Lab group: Birt/Whitley lab
Updated by: Nicole Cray 8-2-13

The following SOP outlines the method for qRT-PCR following cDNA synthesis using the GeneAmp 5700

Bio-Rad iQ SYBR Green Supermix (BioRad, Hercules, CA).

12.5 ul SYBR Green Supermix
10 ul Nuclease free water
0.5 ul F primer (10pmol/ul)
0.5 ul R primer (10pmol/ul)
1.5 ul cDNA sample (1200-1500ng/ml) → NC does not dilute cDNA due to low reads

Blank samples include every ingredient except for the cDNA sample. The 1 ul difference is made up by
adding 1ul additional nuclease-free water.

qRT-PCR protocol:
95C 3 min
95C 15s
62C 30s Repeat for 40 cycles (Note: 62 and not 60 here)
72C 30s
95C 1 min
55C 1 min
Hold at 55C

How to dilute primer:
1. Spin down primers to get them all at the bottom of the tube.
2. Check specifications on primer tubes—usually 100nmol
   Ex. If the tube says “a nmol=b g”, then add 10*a ul TE buffer to dilute to 100pmol
   → 85.5nmol=0.83ng, then add 855 ul TE buffer to get to 100pmol
3. Further dilute to 10pmol for qRT-PCR with RNase-free water (20ul sample to 180ul RNase-free water)